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KINASE CASCADES IN THE REGULATION OF GLUCOSE HOMEOSTASIS

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ABSTRACT

Therapeutic strategies to treat Type 2 Diabetes Mellitus (T2DM) aim at improving muscle insulin sensitivity by either directly modulating, or bypassing defective insulin signaling. Insulin induces metabolic and gene regulatory responses via PI 3-Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) signaling pathways, respectively. MAPK signaling pathways can also be activated by insulin-independent stimuli such as exercise/contraction and stress/hypoxia. AMP-activated Protein Kinase (AMPK) also transduces signals to glucose transport and gene regulatory responses by insulin-independent stimuli. Identifying the molecular mechanisms underlying insulin -dependent and -independent signaling pathways will reveal molecular targets for the pharmacological treatment of insulin resistance.

Glucose induces insulin resistance in skeletal muscle. Glucose sensitive targets in the PI3K signaling pathway were identified in Wistar and Goto-Kakizaki (GK) rats exposed to a 3-hr hyperglycemic infusion. In skeletal muscle, glucose directly activates Phosphoinositide Dependent Kinase 1 (PDK-1) and downstream Protein Kinase C (PKC) isoforms (α/β, δ and ζ) through a mechanism that is independent of changes in PI3K, Protein Kinase B (PKB) and Extracellular signal-Regulated protein Kinase (ERK) phosphorylation. This may account for defects in insulin signaling in non-obese diabetic GK rats, whereby PKCζ was also activated by hyperglycemia. Thus, hyperglycemia acutely modulates signal transduction and this may account for skeletal muscle insulin resistance at the level of the PI3K pathway.

MAPK activation in skeletal muscle from insulin resistant *ob/ob* mice was measured to determine if insulin-dependent and -independent induction of MAPK signaling is intact in insulin resistant tissue. Insulin action on MAPK was impaired in *ob/ob* mice, whereas contraction-mediated effects were preserved. In addition Phorbol 12-Myristate-13-Acetate (PMA) elicited divergent effects on MAPK signaling in *ob/ob* mice; c-Jun NH2-terminal Kinase (JNK) and ERK phosphorylation were preserved, whereas p38-MAPK phosphorylation was refractory. Thus appropriate MAPK response can be elicited in insulin-resistant skeletal muscle via insulin-independent mechanisms. Exercise and muscle contraction may circumvent aberrant MAPK signaling in insulin-resistant skeletal muscle.

Exercise- and hypoxia-stimulated AMPK activity and glucose transport were measured in skeletal muscle from insulin resistant obese Zucker *fa/fa* rats. An isoform-specific defect in AMPKα1 activity in response to contraction was observed, which was inconsequential to glucose transport. Thus, AMPK signaling to glucose transport is unaffected by insulin resistance.

Constitutive activation of AMPK can be achieved by AMPKγ3 missense mutation (R225Q). Transgenic mice overexpressing the R225Q γ3 mutant form were challenged with a 55% fat diet. Transgenic mice have increased lipid oxidation in the presence of lipid oversupply, which attenuates the expected accumulation of intramuscular triglyceride (TG) content and protects against skeletal muscle insulin resistance. In contrast, ablation of AMPKγ3 resulted in increased TG content and impaired insulin action. Thus, the muscle specific AMPKγ3-subunit is a putative target for pharmacological treatment of diet-induced skeletal muscle insulin resistance.

In conclusion insulin signaling defects at the level of PI3K and MAPK pathways appear to contribute to skeletal muscle insulin resistance. Insulin-independent pathways via MAPK and AMPK may prove valuable in the treatment of insulin resistance in T2DM.

Key Words: skeletal muscle, insulin resistance, T2DM, hyperglycemia, high fat diet, glucose transport, PI3K-, MAPK- and AMPK-signaling pathways, AMPK$\gamma 3^{R225Q}$ mutation.

RESUME

Une des stratégies thérapeutiques de traitement du diabète de type 2 consiste à lutter contre l'insulino-résistance musculaire en ciblant directement des défauts de stimulation du transport du glucose par la voie de signalisation à l'insuline ou par des voies parallèles déclenchées par la contraction musculaire ou le stress. La liaison de l'insuline à son récepteur musculaire allume des cascades de signalisation qui transmettent à la fois des signaux métabolique et mitogénique, grâce aux voies de la PI 3-Kinase et des Mitogen-Activated Protein Kinase (MAPK). Les stimuli non insuliniques, tels que le stress ou la contraction musculaire, activent les voies des MAPK et de l'AMP-activated Protein Kinase (AMPK) qui stimulent le transport du glucose et/ou des réponses mitogéniques. L'identification et la compréhension des mécanismes moléculaires qui sous-tendent l'activation de ces cascades de signalisation par des stimuli insuliniques ou non permettront de révéler des cibles thérapeutiques de traitement de l'insulino-résistance musculaire.

L'hyperglycémie est une cause majeure de développement de l'insulino-résistance musculaire. Nous avons identifié des cibles sensibles au glucose en exposant des rats Wistar contrôles et diabétiques Goto-Kakizaki (GK) à une hyperglycémie aiguë. Dans le muscle squelettique, les kinases PDK-1 et PKC α/β, δ et ζ sont activées directement par la glycémie selon un mécanisme indépendant de l'activation des kinases PI3K, PKB et ERK. Chez les rats GK, la PKCζ est aussi activée par une hyperglycémie aiguë. Dans le muscle, le niveau de la glycémie module donc l'activité de protéines de signalisation et cela pourrait expliquer les défauts dans la voie de signalisation de la PI3K.

Pour déterminer si la voie des MAPK est intacte dans un modèle d'insulino-résistance musculaire, nous avons mesuré l'état d'activation de kinases comme JNK, p38-MAPK et ERK dans le muscle squelettique des souris obèses *ob/ob* en réponse à des stimuli insulino-sensibles ou non. Chez ces souris, la réponse insulinique des MAPK est défectueuse alors qu'elles sont normalement activées par la contraction musculaire. De même l'activation par le Phorbol 12-myristate-13-acetate (PMA) de la p38-MAPK est inexistante alors que celle de JNK et ERK est intacte. Ces défauts dans la cascade de signalisation des MAPK pourraient participer à l'insulino-résistance musculaire des souris *ob/ob*. Cependant des stimuli tels que le PMA et la contraction musculaire seraient susceptibles de combattre ces défauts.

Nous avons également étudié le rôle potentiel de sous unités de l'AMPK dans deux modèles de résistance à l'insuline. L'effet de l'hypoxie et de la contraction musculaire sur le transport du glucose a été mesuré dans un modèle de rats Zucker *fa/fa*. Nous avons identifié un défaut d'activation de l'isoforme $\alpha 1$ de l'AMPK en réponse à la contraction musculaire dans le muscle squelettique de ces rats, sans altération du transport du glucose. Ainsi l'activation du transport du glucose par l'AMPK est intacte dans le muscle squelettique de ce modèle animal. Par ailleurs, le rôle de la sous unité $\gamma 3$ de l'AMPK a été évalué chez des souris nourries avec un régime hyperlipidique et qui surexpriment dans le muscle squelettique, une forme constitutivement active de cette sous unité $\gamma 3$. Ces souris montent une capacité accrue d'oxydation des lipides, réduisant l'accumulation de triglycérides intramusculaires et prévenant le développement de l'insulino-résistance. Par contre, les souris dont le gène $\gamma 3$ a été invalidé sont résistantes à l'insuline, et stockent autant de triglycérides que les souris témoins au même régime hyperlipidique. Ainsi la sous-unité $\gamma 3$ de l'AMPK est une cible potentielle pour lutter contre l'insulino-résistance musculaire induite par un régime hyperlipidique.

En conclusion, l'insulino-résistance musculaire peut s'expliquer par des défauts de signalisation dans les voies de la PI3K et des MAPK. L'activation des cascades de signalisation de l'AMPK et des MAPK en réponse à des stimuli, tels que la contraction musculaire ou le PMA, offrent des possibilités de combattre cette résistance à l'insuline.

Mots-clés : muscle squelettique, insulino-résistance, diabète de type 2, hyperglycémie, régime hyperlipidique, transport du glucose, voie de signalisation des PI3K, MAPK et de l'AMPK, mutation AMPK$\gamma 3^{R225}$.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals, and additional unpublished work.

I. Steiler T.L., Galuska D., Leng Y., Chibalin A.V., Gilbert M. and Zierath J.R. Effect of hyperglycemia on signal transduction in skeletal muscle from diabetic Goto-Kakizaki rats. *Endocrinology* 144: 5259-5267, 2003.

II. Leng Y., Steiler T.L. and Zierath J.R. Effects of insulin, contraction and phorbol esters on mitogen-activated protein kinase signaling in skeletal muscle from lean and *ob/ob* mice. *Diabetes* 53: 1436-1444, 2004.

III. Barnes B.R.*, Ryder J.W.*, Steiler T.L., Fryer L.G.D., Carling D. and Zierath J.R. Isoform-specific regulation of 5' AMP-activated protein kinase in skeletal muscle from obese Zucker (*fa/fa*) rats in response to contraction. *Diabetes* 51: 2703-2708, 2002.

IV. Barnes B.R.*, Marklund S.*, Steiler T.L.*, Walter M., Hjälm G., Amarger V., Mahlapuu M., Leng Y., Johansson C., Galuska D., Lindgren K., Åbrink M., Stapleton D., Zierath J.R. and Andersson L. The 5'-AMP-activated protein kinase γ3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J. Biol. Chem.* 279: 38441-38447, 2004.

CONTENTS

ABBREVIATIONS

ACC	Acetyl CoA carbonylase
AICAR	5-aminoimidazole-4-carboxamide riboside
AMPK	AMP-activated protein kinase
AMPKK	AMP-activated protein kinase kinase
AMP/ATP	Adenosine-mono-/tri-phosphate
AS160	*Akt* substrate of 160 kDa
BSA	Bovine serum albumine
bw	body weight
DAG	Diacylglycerol
EDL	Extensor digitorum longus
ERK	Extracellular signal-regulated protein kinase
FFA	Free fatty acid
GK Rat	Goto-Kakizaki rat
GLUT4	Glucose transporter 4
GS	Glycogen synthase
IR	Insulin receptor
IRS	Insulin receptor substrate
JNK	c-jun NH2-terminal kinase
KHB	Krebs henseleit buffer
MAPK	Mitogen-activated protein kinase
MCD	Malonyl CoA decarboxylase
MEK	MAPK kinase
MEKK	MAPK kinase kinase
PDK-1	Phosphoinositide dependent kinase-1
PH	Pleckstrin homology
PI	Phosphatidylinositol
PI 3-Kinase	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
c, n, aPKC	conventionnal, novel, atypical protein kinase C
PMA	Phorbol 12-myristate-13-acetate
$Prkag3^{-/-}$	AMPKγ3 knock out mouse model
PS	Phosphatidylserine
RIA	Radioimmunoassay
SH2	Src homology domain 2
TBST	Tris buffer saline tween
TG	Triglyceride
$Tg\text{-}Prkag3^{wt}$	Transgenic mouse overexpressing wild type AMPKγ3
$Tg\text{-}Prkag3^{225Q}$	Transgenic mouse overexpressing mutant (R225Q) AMPKγ3
T2DM	Type 2 diabetes mellitus

INTRODUCTION

The number of patients diagnosed with Type 2 Diabetes Mellitus (T2DM) is growing at an astronomical rate. Insulin resistance is a characteristic feature of the disease, and leads to altered whole body glucose homeostasis (DeFronzo et al. 1992) and impaired skeletal muscle glucose transport (Zierath et al. 1994; Rothman et al. 1995). Insulin-induced glucose transport in skeletal muscle is mediated by transmembrane glucose transport that occurs in response to Glucose Transporter 4 (GLUT4) translocation from an intracellular pool to the plasma membrane. While GLUT4 expression in T2DM patients is unchanged compared to controls (Garvey et al. 1992; Andersen et al. 1993), insulin-stimulated GLUT4 content in plasma membrane and GLUT4 translocation in skeletal muscle from insulin resistant diabetic patients are reduced (Zierath et al. 1996; Garvey et al. 1998). Therefore the defects along the glucose transport process might lie in the insulin signaling pathway (Vogt et al. 1992; Garvey et al. 1998).

Insulin Signaling Pathways

Glucose transport, activation of glycogen or protein synthesis and initiation of specific gene transcription are regulated via intracellular signaling pathways that are activated when insulin binds to its receptor. Phosphatidylinositol 3-Kinase (PI3K) and Mitogen Activated Protein Kinase (MAPK) pathways are the two major pathways activated in response to insulin receptor phosphorylation.

The insulin receptor (IR) belongs to a large family of growth factor receptors with intrinsic tyrosine kinase activity and it is composed of two α and two β subunits. Insulin binds to the extracellular α-subunits, whereas transmembrane β-subunits contain the tyrosine kinase activity. Following insulin stimulation, IR undergoes autophosphorylation on multiple tyrosine residues (Kasuga et al. 1983), leading to receptor kinase activation and subsequent tyrosine phosphorylation of a family of IR substrate (IRS) proteins.

IRS are commonly referred to as docking proteins, since several other proteins bind to phosphorylated IRS (White 1997). To date, four different IRS molecules have been cloned and the predominant isoforms expressed in skeletal muscle are IRS-1 and IRS-2 (White 1997). IRS are involved in transducing metabolic and mitogenic effects of insulin (Araki et al. 1994, Withers et al. 1998). IRS are phosphorylated on multiple tyrosine residues by IR tyrosine kinase at YXXM and YMXM motifs. Once phosphorylated, IRS serve as docking sites for molecules containing Src homology 2 domains (SH2), namely PI3K and the MAPK activator, Grb2.

The PI3K Signaling Cascade

PI3K is a lipid kinase that associates with IRS-1 and signals to glucose transport following insulin stimulation (Cheatham et al. 1994). PI3K consists of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit with two SH2 domains. Binding of the SH2 domains with the two YMXM motifs present in IRS-1, markedly stimulates PI3K activity, as measured in anti-IRS-1 or anti-phosphotyrosine immunoprecipitates (Herbst et al. 1995). PI3K phosphorylates phosphatidylinositol (PI), PI 4-phosphate and PI 4,5-bisphosphate at position D-3 of the inositol ring

generating respectively PI 3-phosphate, PI 3,4-bisphosphate (PIP_2) and PI 3,4,5-triphosphate (PIP_3).

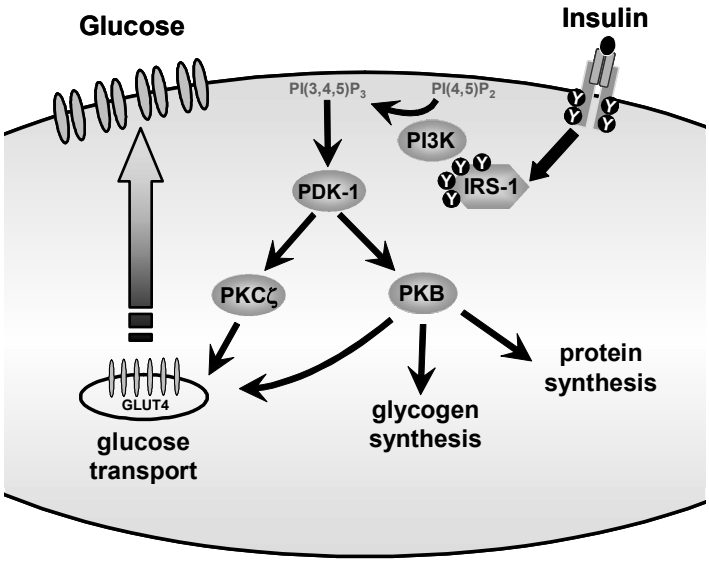


Figure 1. IRS-1 signaling cascade to glucose transport

PIP_3 is an allosteric activator of Phosphoinositide-Dependent Kinase 1 (PDK-1). PDK-1 is a serine/threonine kinase that activates Protein Kinase B (PKB) and Protein Kinase C (PKC) via direct phosphorylation of key sites in their activation loop (Mora et al. 2004). PDK-1 is rate-limiting for insulin-stimulated glucose transport and subsequent GLUT4 translocation (Bandyopadhyay et al. 1999; Grillo et al. 1999). Activation of PDK-1 remains controversial, mainly because conclusions have been drawn from cell culture models and *in vivo* data is lacking. In resting cells, PDK-1 appears to be constitutively active (Mora et al. 2004). However additional insulin-stimulated serine (Casamayor et al. 1999) and tyrosine (Park et al. 2001) phosphorylation events may occur that seem to be associated with substantial increases in PDK-1 activity (Chen et al. 2001). Recruitment of PDK-1 to the plasma membrane also seems to be necessary for full activation of the enzyme, a finding consistent with insulin-stimulated plasma membrane translocation of downstream PDK-1 substrates (Egawa et al. 2002).

The downstream pathways by which insulin-stimulated PI3K activity leads to GLUT4 translocation involve activation of the serine/threonine kinases PKB and PKC. Several lines of evidence indicate that PKB functions downstream of PI3K (Burgering et al. 1995; Kohn et al. 1996; Franke et al. 1997) and mediates insulin signaling to glucose transport (Cho et al. 2001; Jiang et al. 2003, Katome et al. 2003). PKB is encoded by *Akt* proto-oncogene and is defined by an NH_2-terminal regulatory domain and a pleckstrin homology (PH) domain required for protein-protein interaction (Downward 1995). To date, three PKB isoforms have been cloned. PKBα encoded by *Akt1,* and PKBβ encoded by *Akt2,* are the predominant isoforms expressed in skeletal muscle (Hanada et al. 2004). Binding of PI3K products to the PH domain results in translocation of PKB to the plasma membrane, where it is activated through phosphorylation by upstream kinases including PDK-1. Full activation of PKB requires phosphorylation of threonine 308 and serine 473 by PDK-1 (Alessi et al. 1996) and leads to its homodimerisation (Hanada et al. 2004).

PKBβ plays a major role in regulating glucose homeostasis and insulin-stimulated glucose transport in EDL (Cho et al. 2001). Once activated, PKB phosphorylates downstream substrates, such as the newly identified Akt Substrate of 160 kDa (AS160). AS160 is a 160 kDa protein that is activated and redistributed to the plasma membrane following phosphorylation by PKB (Kane et al. 2002). In adipocytes, AS160 mediates insulin-stimulated PKB signaling to glucose transport possibly via regulation of GLUT4 endocytosis (Sano et al. 2003; Zeigerer et al. 2004). AS160 is also expressed in rat skeletal muscle and phosphorylated in response to insulin and contraction (Bruss et al. 2005), suggesting a potentially similar role in this tissue. In addition to glucose transport, PKB mediates the effect of insulin on glycogen synthesis via glycogen synthase kinase 3 phosphorylation (Markuns et al. 1999), and lipid metabolism via phosphodiesterase 3B phosphorylation (Wijkander et al. 1998).

Other downstream candidates in the insulin signaling pathway to glucose transport include members of the PKC family. PKC are important regulators of cell growth, differentiation and metabolism (Hug et al. 1993; Dekker et al. 1994; Bandyopadhyay et al. 1997). The PKC family consists of at least 12 highly conserved and ubiquitously expressed serine/threonine kinase isotypes that are classified into three subfamilies on the basis of their enzymatic properties, including conventional cPKC (α, β and γ), novel nPKC (δ, ε, η and θ) and atypical aPKC (λ and ζ) isoforms (Newton 1995). All PKC isoforms are expressed in skeletal muscle (Puceat et al. 1996). PKC are characterized by their dependence upon lipids for activity. Specifically, the conventional and novel isotypes display a physiological role for diacylglyceride (DAG), which is necessary for translocation of PKC to cellular membranes (Newton 1995). Phorbolester can substitute for DAG in stimulating and prolonging PKC activity (Nishizuka 1995). In addition to DAG regulation, all PKC isozymes require phosphatidyl serine (PS), an acidic lipid located exclusively on the cytoplasmic face of membranes, whereas nPKC require Ca^{2+} for optimal activity (Newton 1995). All PKC isoforms can be activated by PDK-1 via phosphorylation on specific serine/threonine residues in the activation loop (Mora et al. 2004). To date, the isoform specific function of PKC isoforms and the potential role of these kinases in T2DM pathogenesis remain largely undefined.

PKC positively and negatively mediate insulin action via isoform specific activation. Indeed PKC-δ and -ζ are implicated in acute insulin-stimulated glucose transport (Bandyopadhyay et al. 1997; Kotani et al. 1998, Braiman et al., 1999), whereas PKC-α and -β act as negative feedback regulators of insulin signaling (Standaert et al. 1999; Letiges et al. 2002). Recent evidence suggests there is an isoform specific role of PKC isoforms in the regulation of insulin action. The PKCδ-isoform is involved in skeletal muscle insulin-stimulated glucose transport via direct interaction with the IR (Braiman et al. 2001), independently from PI3K, while the PKCζ-isoform mediates insulin-stimulated glucose transport through a PI3K-dependent mechanism (Standaert et al. 1996, Ishizuka et al., 2001). PKCζ has also been hypothesized to stimulate glucose transport by an insulin-independent pathway (Hansen et al. 1997). Indeed physiological exercise (Perrini et al. 2004), the adenosine analogue 5-aminoimidazole-4-carboxamide riboside (AICAR) and phorbolester activate PKCζ, possibly via MAPK-(Chen et al. 2002) and/or AMP-activated protein Kinase (AMPK)-mediated mechanisms (Chen et al. 2002).

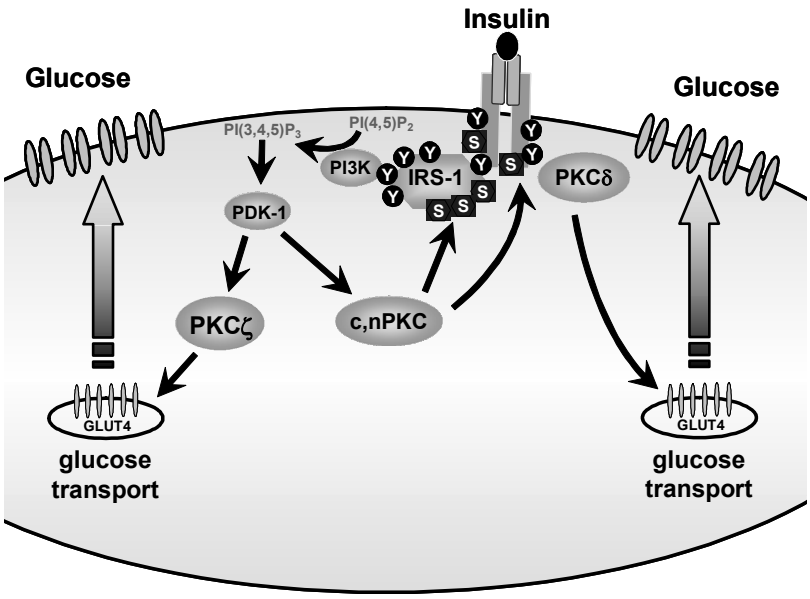


Figure 2. PKC isoform-specific regulation of insulin signaling
Insulin regulation of glucose transport is mediated by PKC, which elicit isoform-specific opposing effects on glucose transport. Insulin activation of PKC-δ and -ζ upregulates GLUT4 translocation to the plasma membrane and subsequent glucose transport activity whereas insulin activation of c,nPKC downregulates the process.

PKC attenuation of insulin signaling involves PKC-induced serine phosphorylation of IR and IRS-1 (Bollag et al. 1986; Chin et al. 1993). Increased IR and IRS-1 serine phosphorylation results in reduced IRS-1 tyrosine phosphorylation and PI3K activity following insulin stimulation (Newton 1995, Li et al., 2004). Increased IR and IRS-1 serine phosphorylation rapidly suppresses insulin-stimulated glucose transport (Tanti et al. 1994). Thus PKC appear to attenuate insulin action via IR desensitization under normal physiological conditions. This may be of particular importance in the case of T2DM and obesity where insulin resistance has been associated with aberrant PKC activation.

The PI3K signaling pathway plays a key role in transmitting insulin signaling to glucose transport in skeletal muscle. In humans and rodents, insulin-stimulated glucose uptake occurs in response to GLUT4 translocation from an intracellular pool to the plasma membrane (Guma et al. 1995). GLUT4 is the major glucose transporter isoform expressed in skeletal muscle (Charron et al. 1989) and it catalyzes a rate limiting step for glucose uptake and metabolism (Marshall et al. 1994). In light of these results, functional defects in any of the IRS-1/PI3K signal transducers may account for impairments in glucose uptake and metabolism in diabetic skeletal muscle.

The MAPK Signaling Cascade

MAPK are conserved and ubiquitously expressed serine/threonine kinases involved in the transduction of externally derived signals regulating cell growth, division, differentiation, adaptation and apoptosis. Mammalian cells contain at least three major classes of MAPK: the Extracellular signal Regulated Kinases 1 and 2 (ERK1/2), c-jun N-terminal Kinase (JNK) and p38-MAPK. MAPK activation is regulated via a series of parallel kinase cascades organized into multiproteic

complexes, with each pathway comprising three kinases that successively phosphorylate and activate the downstream component. In the ERK1/2 kinase pathway, the proximal Raf-1 (a MAPK Kinase Kinase or MEKK) phosphorylates and activates MAPK Kinase 1 and 2 (MEK 1 and 2). MEK1 and 2 are dual threonine/tyrosine kinases that in turn phosphorylate and activate ERK1/2. Regulation of the JNK and p38 MAPK modules also occurs via a MEK and MEKK phosphorylation cascade, mimicking that of the ERK1/2 cascade (Pearson et al. 2001).

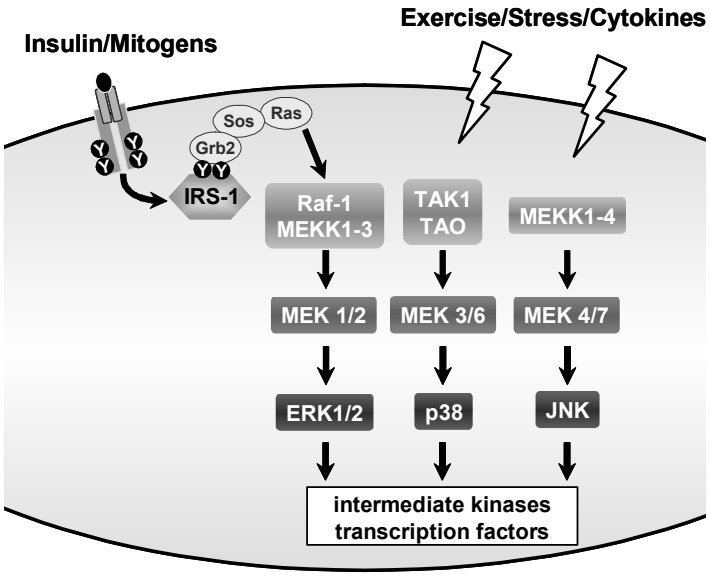


Figure 3. MAPK signaling cascade

MAPK signaling enhances gene expression via phosphorylation of a wide variety of transcription factors and intermediary kinases. Transcription factors can be regulated by all three MAPK or by one in particular. For instance, ERK1/2 and p38-MAPK phosphorylate serum response factor accessory protein-1 (Janknecht et al. 1995, Whitmarsh et al., 1997), whereas all three MAPK phosphorylate Elk-1 (Cavigelli et al. 1995, Kim et al., 1997, Whitmarsh et al., 1997). p38-MAPK phosphorylates Myosin Enhancing Factor 2 (MEF2) (Zhao et al. 1999), while JNK modulates c-jun (Janknecht et al. 1995), Signal Transducer and Activator of Transcription 3 (STAT3) (Lim et al. 1999) and Nuclear Factor Activated T-cell (NFAT) (Chow et al. 2000). STAT3 has recently been implicated in the regulation of hepatic gluconeogenesis (Inoue et al. 2004), whereas MEF2 and NFAT mediate skeletal muscle fiber type (Chin et al. 1998).

Intermediary kinases can also be phosphorylated by one or several MAPK. ERK1/2 and p38-MAPK phosphorylate mitogen- and stress-activated protein kinase-1 and -2 (Deak et al. 1998) and MAPK activated protein kinase-2 and -3 (Cuenda et al. 1995). ERK1/2 specifically modulates p90 ribosomal S6 kinase (Xing et al. 1996), whereas JNK specifically phosphorylates IRS-1(Lee et al. 2003). Activation of the JNK pathway interferes with insulin action via serine phosphorylation of IRS-1 (Aguirre et al. 2000) and subsequent downregulation of tyrosine induced-insulin intracellular signaling (Lee et al. 2003). Thus, disruption of the JNK-mediated IRS-1 serine phosphorylation may be a potential treatment strategy for insulin resistance.

The MAPK signaling cascade allows for transduction and/or amplification of highly specialized signals. MAPK pathways transduce insulin-dependent and -independent signaling to gene regulatory responses. ERK1/2 is traditionally a mitogenic signal transducer, whereas p38-MAPK and JNK also sense stress-related signals. Indeed the ERK1/2 module is mainly activated by hormones including insulin, growth factors, serum; G-protein coupled receptor ligands, and transforming agents, whereas p38-MAPK and JNK modules are also recruited by cytokines, cellular stress such as osmotic shock, heat shock and DNA interfering agents and protein synthesis (Pearson et al. 2001).

Insulin-dependent MAPK signaling

Insulin induces mitogenic responses via activation of all three MAPK modules (Moxham et al. 1996; Somwar et al. 2000). Grb2-mediated ERK1/2 activation is a well- characterized pathway in this regard. Grb2 is a SH2 domain-containing protein that associates with IRS-1 (Skolnik et al. 1993). Following insulin stimulation, Grb2 interacts with Son of sevenless (Sos) via its SH3 domains (Leevers et al. 1994). Recruitment of Sos to the plasma membrane results in Ras GDP↔GTP exchange and subsequent activation (Downward 1996). Once activated, Ras interacts with Raf-1 and turns on MAPK signaling to ERK1/2 (Marais et al. 1995). Insulin activation of MAPK might also occur via a Ras-independent, PKC-dependent mechanism (Formisano et al. 2000; Letiges et al. 2002). In skeletal muscle the upstream MEKK activating mechanism of MAPK remains undefined.

Insulin-independent MAPK signaling

Exercise training has beneficial effects on whole body insulin sensitivity (Houmard et al. 1993; Hughes et al. 1993). In response to regular contractile activity or physical exercise, skeletal muscle undergoes profound adaptive changes in regard to metabolic and structural properties (Eriksson et al. 1973). These changes might be mediated by increased protein expression of GLUT4 and key insulin signaling enzymes (Ren et al. 1995; Chibalin et al. 2000). However the mechanism involved in exercise-induced skeletal muscle adaptation is unclear. MAPK might be intracellular messengers linking skeletal muscle activity to adaptation. Indeed, exercise activates MAPK in human (Aronson et al., 1997b) and rat (Goodyear et al., 1996; Aronson et al., 1997a) skeletal muscle and in cultured cells, MAPK activation results in cell growth and differentiation (Chen et al. 2001).

MAPK signaling and muscle fiber type

Insulin-stimulated glucose transport is positively correlated with slow- vs. fast-twitch skeletal muscle fibers (Hickey et al. 1995). Insulin action in skeletal muscle occurs through fiber-type specific activation of components of the insulin signaling cascade (Song et al. 1999). Skeletal muscle fiber-type transformation is associated with improved insulin sensitivity (Ryder et al. 2003). Moreover MAPK signaling pathways are differentially activated and expressed in slow- and fast-twitch muscle fibers (Wojtaszewski et al. 1999; Wretman et al. 2000). Thus MAPK fiber-type-specific signaling may be of importance in the search for means to improve insulin sensitivity in insulin resistant states. However little to no data is available on muscle fiber type specific mitogenic effects of insulin-dependent and -independent stimuli on MAPK in normal and insulin resistant animal models.

The AMPK Signaling Cascade

Exercise/contraction and stress/hypoxia are insulin-independent stimuli that also signal to glucose transport. Understanding the molecular mechanisms underlying insulin-independent activation of muscle glucose transport might reveal potential therapeutic targets for the treatment of insulin resistance. AMPK was recently identified as a key mediator of exercise-stimulated glucose transport (Hayashi et al. 1998; Musi et al. 2001). In skeletal muscle, AMPK monitors the energy status of the cell by sensing changes in AMP:ATP and Creatine:Cr-P ratios. Thus energy depleting stimuli, such as exercise and contraction or pathophysiological hypoxia, activate AMPK. The resulting effect is energy repletion via stimulation of glucose transport and fuel utilization through increased fatty acid oxidation and inhibition of fat storage (Winder 2001).

AMPK is a heterotrimeric complex comprising a catalytic α-subunit and two regulatory β- and γ-subunits. Two isoforms encode the α- and β-subunits, and three isoforms encode the γ-subunit. Although α1, β1 and γ1 are the predominant isoforms in most cells; skeletal muscle also expresses α2, β2, γ2 and γ3 (Kemp et al. 2003). Specific expression of γ3 in glycolytic skeletal muscle fibers (Mahlapuu et al. 2004) provides evidence that the combination of different isoforms may have specific functions in skeletal muscle. Indeed identification of mutated forms of all three γ-subunits shows similarities and differences in the metabolic phenotype (Milan et al. 2000).

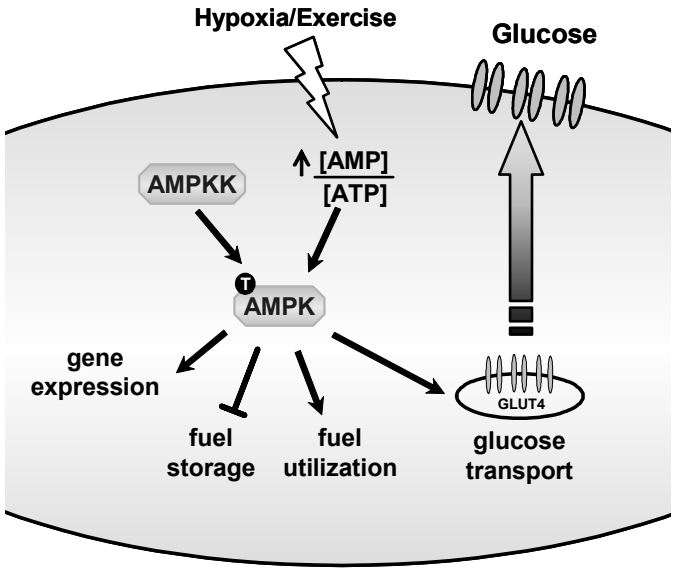


Figure 4. AMPK signaling cascade

Activation of AMPK occurs via both allosteric activation by AMP and phosphorylation of threonine 172 in the activation loop of the catalytic α-subunit by upstream AMPK Kinase (AMPKK). The tumor suppressor LKB1 was recently identified as the major AMPKK (Woods et al. 2003) but dominant-negative expression of LKB1 does not lead to complete loss of basal AMPK activity suggesting that other AMPKK still remain to be identified.

Pharmacological activation of AMPK can be achieved in response to AICAR. AICAR is an adenosine analog that is taken up into cells and converted by adenosine kinase to the monophosphorylated nucleotide 5-aminoimidazole-4-carboxamide-1-D-

ribofuranosyl-5'-monophosphate (ZMP). ZMP mimics all of the activating effects of AMP on the AMPK system, although it is less potent than AMP itself. AICAR stimulates AMPK and increases glucose uptake by promoting GLUT4 translocation to the cell surface in rodent (Musi et al. 2003) and human skeletal muscle (Koistinen et al. 2003). Thus, activation of AMPK may be one strategy to bypass defective insulin signaling to glucose transport.

Numerous downstream targets of AMPK have been identified. These proteins are important for glucose and lipid metabolism and include 6-phosphofructo-2-kinase 2, glycogen synthase (GS), IRS-1, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, hormone sensitive lipase, acetyl-coenzyme A carboxylase (ACC), glycerol phosphate acyl transferase and malonyl CoA decarboxylase (MCD) (Hardie 2003). AMPK regulation of gene involved in fuel metabolism might account for exercise-induced improvements in skeletal muscle insulin sensitivity (Musi et al. 2003).

Targeting AMPK for the treatment of T2DM

AMPK activation causes many metabolic changes that should be beneficial for subjects with T2DM. This is supported by evidence that chronic activation of AMPK via AICAR treatment improves glucose and lipid homeostasis, and insulin resistance in animal models of diabetes and obesity (Iglesias et al. 2002; Olsen et al. 2002). Leptin activates AMPK in skeletal muscle, thereby increasing fatty acid oxidation (Steinberg et al. 2003), whereas adiponectin activates AMPK in liver and muscle to stimulate glucose and fatty acid usage, and to inhibit hepatic glucose production (Tomas et al. 2002; Yamauchi et al. 2002). Furthermore AMPK mediates the action of the antidiabetic drugs metformin (Musi et al. 2002) and rosiglitazone (Fryer et al. 2002), indicating that AMPK activators may play a role as antidiabetic agents. The underlying mechanisms for these effects remain largely undefined. Some evidence suggests that there is cross-talk between AMPK and insulin signaling pathways. Indeed AMPK activation has been observed simultaneously with activation of PKCζ and ERK1/2 under insulin-independent activation of glucose transport (Chen et al. 2002). AMPK also phosphorylate IRS-1 on serine 789 in C2C12 myotubes, which correlates with an increase in IRS-1 associated PI3K activity (Jakobsen et al. 2001).

Type 2 Diabetes Mellitus and Skeletal Muscle Insulin Resistance

Recent studies in animal models (Folli et al. 1993; Saad et al. 1993) and isolated tissues from T2DM patients (Björnholm et al., 1997; Krook et al., 2000) provide evidence that genetic and environmental factors regulate insulin signaling by changing the function, or covalent modifications of proteins involved in the intracellular network of insulin action.

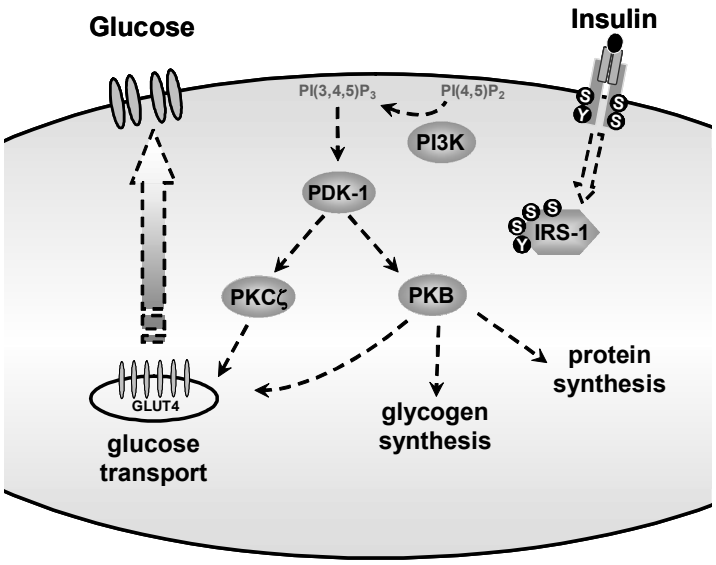


Figure 5. Defects in PI3K signaling

Defects in the early steps of insulin signaling pathways

Several studies report that a decrease in IR and IRS-1 protein phosphorylation is associated with reduced PI3K activity in skeletal muscle from T2DM patients (Björnholm et al., 1997) and obese insulin resistant subjects (Goodyear et al. 1995) However reduced phosphorylation of both IR and IRS-1 was unrelated to changes in protein expression (Björnholm et al., 1997; Zierath et al., 1998). In contrast reduced number and activity of IR has been observed in skeletal muscle and liver from *ob/ob* mice (Kahn et al. 1973; Soll et al. 1975). Genetic analysis also confirms these findings: IRS-1 exhibits multiple natural polymorphisms that are more common in T2DM patients than in control subjects. The G972R for instance, is associated with decreased insulin sensitivity during the oral glucose tolerance test and homozygotes for the codon exhibits diabetes (Almind et al. 1993; Laakso et al. 1994). Further studies revealed a specific defect in binding of the p85 regulatory subunit of PI3K to IRS-1 resulting in reduced IRS-1 associated PI3K activity (Almind et al. 1996). Interestingly subsequent PDK-1 activation is unaltered in T2DM patients (Kim et al. 2003).

Recent evidence suggests that reduced tyrosine phosphorylation of IR and IRS-1 is linked to increased serine phosphorylation of these targets (Tanti et al. 1994; Kanety et al. 1995), thereby providing a mechanism for impaired insulin-stimulated glucose transport (Le Marchand-Brustel et al., 2003). Indeed aberrant serine phosphorylation of IRS-1 has been noted in insulin resistant T2DM patients (Bouzakri et al. 2003) and obese (Eckel et al. 1985) or high fat-fed animal models (Schmitz-Peiffer et al., 1997; Schmitz-Peiffer 2000. Aberrant IRS-1 tyrosine and serine phosphorylation may directly interfere with insulin activation of downstream PI3K and MAPK signaling cascades.

Defects in PI3K signaling

PKB is a downstream target of PI3K (Franke et al. 1995) through PIP_3 and PDK-1 (Alessi et al. 1997). PKB is stimulated in human skeletal muscle in response

to either *in vitro* (Krook et al. 1998) or *in vivo* (Kim et al. 1999) insulin exposure and the activation is sustained throughout the insulin stimulation. Insulin-induced PKB phosphorylation (up to 60 nM) is reduced in skeletal muscle from T2DM patients compared to control subjects (Krook et al. 1998). At lower, more physiological insulin concentrations, PKB phosphorylation and activation is however comparable between T2DM patients and lean controls (Kim et al. 1999), despite severely impaired PI3K and GS activity (Vaag et al. 1992). Aberrant PKB activation is also controversial in insulin resistant animal models (Krook et al. 1997; Kim et al. 2000; Kanoh et al. 2001; Standaert et al. 2004). Thus, the role of PKB in the development of insulin resistance in skeletal muscle remains unclear.

PKC are downstream targets of PI3K and can be activated following insulin stimulation. Insulin activation of PKC isoforms leads to positive and negative modulation of its signal. Aberrant isoform specific PKC translocation, activation and protein expression have been reported in skeletal muscle from insulin resistant animal models. For example, the positive insulin signal transducers, PKC-δ and -ζ, are down-regulated in insulin resistant animal models (Avignon et al. 1996; Schmitz-Peiffer et al. 1997), indicating that these isoforms are candidates leading to impaired glucose uptake and metabolism in diabetes. Similarly in human, insulin-stimulated PKCζ is impaired in skeletal muscle from glucose intolerant, diabetic (Beeson et al. 2003) and insulin resistant obese (Kim et al. 2003) subjects.

A number of studies have linked increased c,nPKC activation in skeletal muscle to diminished insulin sensitivity in humans (Itani et al. 2000) and animals models of T2DM (Schmitz-Peiffer 2000). Isoform specific defects in skeletal muscle have been revealed through the use of PKC isoform-specific antibodies. PKCθ is highly expressed in skeletal muscle (Avignon et al. 1996). In humans, PKCθ activity and distribution is increased in skeletal muscle of obese diabetic compared to obese normoglycemic humans (Itani et al. 2001). In addition PKCθ mRNA is increased in T2DM patients and inversely correlated with insulin sensitivity (Gray et al. 2003). In these studies, all other PKC isoforms were unaltered. In animals models of T2DM, PKC-δ and -θ phosphorylation is reduced in diabetic Goto-Kakizaki (GK) rats and obese *ob/ob* mice (Avignon et al. 1996), whereas PKC-ε, -δ and -θ phosphorylation is increased in the high fat-fed rat model of insulin resistance (Schmitz-Peiffer et al. 1997). Ablation of PKCθ in mice prevents against high fat diet-induced insulin resistance (Kim et al. 2004). The cause of aberrant PKC activation in the development of insulin resistance associated with T2DM is poorly defined. Recent evidence suggests that changes in PKC isoform specific activation can occur in response to increased glucose and/or lipid availability, whereas chronic changes could occur in response to increased glucose and/or insulin concentrations (Schmitz-Peiffer 2000). Thus little is known about the involvement of PKC in insulin signaling to glucose transport in T2DM. Moreover, the distribution and activation of PKC isoforms and the role of these targets on intracellular events in skeletal muscle as related to T2DM is largely unknown. Nevertheless, activation of PKC is likely to be important in the development of diabetic complications (Way et al. 2001).

Defects in MAPK signaling

The effect of T2DM on MAPK signaling in skeletal muscle is incompletely resolved. Activation and expression of p38-MAPK and ERK1/2 in diabetic insulin

sensitive tissues is either aberrant (Koistinen et al. 2003) or unaltered (Krook et al. 2000). In contrast JNK seems to play a key role in the pathogenesis of insulin resistance. JNK is abnormally elevated in various tissues under diabetic conditions (Hirosumi et al. 2002). Moreover JNK knock out mice are protected from obesity-induced insulin resistance (Hirosumi et al. 2002). Therefore JNK inhibition has been proposed as a therapeutic target for the treatment of insulin resistance (Bogoyevitch et al. 2004). Thus little is known of the physiological implications of MAPK regulation in the context of insulin resistant states such as diabetes and obesity.

Glucose-Induced Insulin Resistance

Chronic hyperglycemia is a cardinal feature of diabetes and obesity. Elevation of plasma glucose concentration for 24 hrs in human (Yki-Jarvinen et al. 1987) and 72 hrs in rats (Hager et al. 1991), to pathophysiologically relevant glucose concentrations (10-40 mM) markedly suppresses whole body insulin-simulated glucose uptake. In addition, selective correction of hyperglycemia normalizes insulin sensitivity (Rossetti et al. 1987), providing compelling evidence that hyperglycemia directly causes insulin resistance.

The mechanism by which hyperglycemia decreases whole body glucose uptake is largely localized to skeletal muscle tissue, and can be explained by reduced muscle glucose utilization and metabolism, particularly glycogen biosynthesis (Yki-Jarvinen et al. 1990). The hyperglycemia-induced impairments in insulin action on skeletal muscle glucose utilization are mediated by defects in glucose transport (Rossetti et al. 1987) and reduced plasma membrane GLUT4 transporter number (Dimitrakoudis et al. 1992). Hyperglycemia-induced defects at different steps of the insulin signaling pathway to glucose transport have also been identified.

Hyperglycemia-induced impairments in PI3K signaling

Hyperglycemia appears to impair the insulin signaling step between PI3K and PKB activation. Isolated EDL muscle incubated for 4-hr with 20 mM glucose exhibit reduced insulin-stimulated glucose uptake and metabolism. In addition insulin-stimulated PKB activation is impaired whereas PI3K and MAPK activities are unchanged (Kurowski et al. 1999). In contrast, post-receptor insulin signaling is increased at the level of IR, IRS-1 and PI3K in parallel with impaired PKB activation in soleus muscle from streptozotocin-injected rats (Oku et al. 2001). Amelioration of glycemia in these rats normalizes PKB activity. Similarly correction of hyperglycemia in the non obese diabetic GK rat normalizes PKB activation and glucose transport (Song et al. 1999) suggesting that impaired PKB activation underlies glucose-induced insulin resistance in skeletal muscle. Interestingly, the toxic effects of glucose are prevented by adding AICAR to the media (Tomas et al. 2002).

Lipid-Induced Insulin Resistance

Management of dyslipidemia is of equal importance to control of hyperglycemia in the care of patients with T2DM. Hyperlipidemia *per se* is an independent primary cause of insulin resistance. Healthy volunteers subjected to

artificial elevation of plasma free fatty acid (FFA) or triglyceride (TG) develop whole body insulin resistance after 4-hr of intralipid infusion (Boden et al. 1995; Roden et al. 1996; Brechtel et al. 2001) and, 2-hr later, both oxidative glucose disposal and muscle glycogen synthesis is diminished. High fat feeding also leads to impaired glucose transport (Grundleger et al. 1982), as well as defects in GLUT4 recruitment to the plasma membrane (Zierath et al. 1997) and expression, in very high fat diets (Leturque et al. 1991, Kahn et al. 1993). Several studies have investigated alterations in insulin signaling in high fat-fed rodents. Insulin resistance has been observed together with diminished IRS-1 tyrosine phosphorylation (Zierath et al. 1997; Anai et al. 1999), PKB and PKCζ activation (Tremblay et al. 2001; Kim et al. 2002).

Mechanisms of hyperlipidemia-induced insulin resistance

Hyperlipidemia might directly modulate PI3K and MAPK signal transducers activation via lipid intermediates. Long chain fatty acid Coenzyme A, DAG and FFA directly activate PKC (Schmitz-Peiffer 2000). FFA also activate JNK (Gao et al. 2004), whereas ceramide specifically interacts with PKB (Schmitz-Peiffer et al. 1999). FFA activation of PKCθ provides a mechanism for FFA-induced insulin resistance (Griffin et al. 1999) via serine phosphorylation (Li et al. 2004) and degradation of IRS-1 (Gao et al. 2004). FFA activation of JNK promotes insulin resistance via association with and phosphorylation of, IRS-1 on serine 307 (Aguirre et al. 2000) which blocks interaction with the IR and inhibits insulin action (Aguirre et al. 2002).Thus, nutrient oversupply can contribute to diminished insulin signaling at early and intermediate steps of the PI3K signaling pathway. These effects may be mediated by aberrant PKC activation, following intracellular accumulation of glucose and lipid intermediates. Prevention of intracellular accumulation of these intermediates might improve skeletal muscle insulin sensitivity.

Strategies to prevent skeletal muscle insulin resistance

Skeletal muscle insulin resistance is an important pathophysiological feature of T2DM and therefore a major site of action for therapeutic strategies. One approach is to directly modulate, or bypass defective insulin signaling, with the ultimate goal to increase GLUT4 translocation to the plasma membrane and glucose transport. To determine whether these molecules are potential sites of pharmacological intervention to overcome skeletal muscle insulin resistance mechanism of action requires validation. Understanding the molecular mechanisms underlying defective insulin signaling and exercise/stress signaling in genetically or environmentally-induced insulin resistant animal models may prove valuable to the biological validation of diabetes prevention targets.

AIMS

The primary aim of this thesis was to investigate the activation of intracellular signaling pathways following insulin-dependent and -independent stimuli in skeletal muscle from insulin resistant diabetic and/or obese rodents. Defects in insulin-stimulated PI3K and MAPK activation will be further explored in parallel with MAPK and AMPK responses to muscle contraction and stress. AMPK is hypothesized to be a potential therapeutic target for the treatment of insulin resistance and T2DM. Thus the role of the muscle specific AMPKγ3-subunit in regulating glucose homeostasis and preventing diet-induced insulin-resistance will be determined using AMPKγ3 transgenic and knock out mice.

This thesis will address the following questions:

- Does acute hyperglycemia lead to skeletal muscle insulin resistance in Wistar and non obese diabetic Goto-Kakizaki rats?
- Does impaired MAPK signaling contribute to skeletal muscle insulin resistance in obese diabetic *ob/ob* mice?
- What is the effect of insulin-independent stimuli on skeletal muscle MAPK signaling in lean and obese *ob/ob* mice?
- Is AMPK signaling intact in obese diabetic *fa/fa* rats?
- Is the AMPKγ3-isoform a therapeutic target for the treatment of insulin resistance?

EXPERIMENTAL PROCEDURES

Animal Models of Insulin Resistance Used in This Thesis

The Goto-Kakizaki rat model

The GK rat is a non-obese animal model of T2DM. The model was developed by selective breeding over 35 generations of glucose intolerant Wistar rats (Goto et al. 1976; Goto et al. 1988). GK rat exhibit mild hyperglycemia, glucose intolerance, impaired insulin secretion and excessive hepatic glucose production (Picarel-Blanchot et al. 1996). Moreover decreased hepatic insulin sensitivity and moderate insulin resistance in skeletal muscle and adipose tissue have also been described (Bisbis et al. 1993). The GK rat serves as an ideal model to study diabetes without the contribution of dyslipidemia or obesity.

The ob/ob mouse model

The *ob/ob* mouse is characterized by a spontaneous mutation of the leptin gene which prevents secretion of mature leptin and leads to hyperphagia, reduced energy expenditure, hyperglycemia, hyperinsulinemia, severe glucose intolerance, and early-onset morbid obesity (Mordes et al. 1981; Pelleymounter et al. 1995; Chua et al. 1996). The mutation in the leptin gene occurs in a single base pair (C428T) that results in premature termination of leptin synthesis (Coleman 1978). Leptin administration to *ob/ob* mice normalizes all aspects of the obesity and diabetes syndrome (Halaas et al. 1995).

The Zucker fa/fa rat model

Obese Zucker rats have a spontaneous mutation in the extracellular domain of the leptin receptor and exhibit hyperphagia, dyslipidemia, impaired glucose tolerance, and morbid obesity (Chua et al. 1996). The *fa/fa* mutation observed in Zucker rats is an A880C nucleotide missense mutation resulting in reduced cell surface leptin receptor expression and leptin resistance (Yamashita et al. 1997; Crouse et al. 1998; da Silva et al. 1998).

The AMPKγ3 knock out (Prkag3$^{-/-}$) and transgenic (Tg-Prkag3) mice models

AMPK is a key mediator of exercise-stimulated glucose transport and fatty acid oxidation (Carling 2004). A spontaneous missense mutation in AMPKγ3 subunit resulting in the substitution of arginine 225 by glutamine (R225Q) was identified in Hampshire pigs, and shown to be associated with increased skeletal muscle glycogen content (Milan et al. 2000). This suggests that AMPKγ3 might play a role in regulating glucose homeostasis. Transgenic mice specifically overexpressing wild type (Tg-Prkag3^{wt}) or R225Q mutated (Tg-Prkag3^{225Q}) forms of AMPKγ3 in white muscle, as well as AMPKγ3 knock out mice (Prkag3$^{-/-}$) were generated using the classical targeted mutagenesis technology. Prkag3$^{-/-}$, Tg-Prkag3^{wt} and Tg-Prkag3^{225Q} mice were phenotyped for the metabolic consequences of genetic modification of AMPKγ3 expression in skeletal muscle.

Environmentally-induced insulin resistance: high fat feeding

Insulin resistance can be rapidly induced in control rats and mice fed a high fat diet for a short period of time (Storlien et al. 1986, Hedeskov et al. 1992, Zierath et al. 1994). Combined euglycemic-hyperinsulinemic clamp and tracer studies reveal that high fat diet-induced insulin resistance first occurs in the liver followed by widespread impairment in insulin action in skeletal muscle and adipose tissues. High fat-fed rat exhibit hyperphagia, reduced energy expenditure, increased adiposity, glucose intolerance, hyperglycemia, hyperinsulinemia and dyslipidemia (Kraegen et al. 1986; Storlien et al. 1993). High fat diet-induced insulin resistance is a flexible approach that allows studying progressive development of the metabolic syndrome (Storlien et al. 1996). The degree of the metabolic defect is proportional to the duration of the diet. In addition, the percentage of fat in the diet also influences the degree of the metabolic defect. AMPK transgenic and knockout mice were placed on a high-fat diet as described below.

Animal Care

GK rats (200-250 g) were purchased from the Department of Endocrinology, Karolinska Hospital (Stockholm, Sweden). Wistar control rats, C57/Bl6 control mice and *ob/ob* mice (12-16 weeks old) were purchased from B&K Universal AB (Sollentuna, Sweden). Zucker (*fa/fa*) rats and lean littermates (11-12 weeks) were purchased from Charles River (Uppsala, Sweden). Transgenic and knock-out AMPKγ3 mice (8-16 weeks old) were generated as described in paper IV by the Department of Animal Breeding and Genetics, Uppsala Biomedical Center (Uppsala, Sweden). Mice genotyping was performed on tail biopsies from 3 week-old transgenic and knock-out animals. DNA was first extracted using a DNA extraction kit (Qiagen, Hilden, Germany), then amplified by PCR using the primer sequences in table 1, and finally identified on a 4% agarose gel using a DNA ladder (Fermentas GmbH, St. Leon-Rot, Germany).

Table 1. AMPK primer sequences

PRIMERS	AMPKγ3 KNOCK OUT	AMPKγ3 OVEREXPRESSOR
Forward	TGGTTGGTGCTCTTTCAGAGGACCCAG	AAGATGGCTTGGGTGTGAGGAC
Forward	TTGGCAAATGGACAAATCTGGAGCTAGG	GCTGCCCAGCAAACCTAAAC
Reverse	GCTGCTAAAGCGCATGCTCCAGAC	TGCTCCGATTGATCAGTTCC

Rodents were matched for age, sex and body weight. All animals were housed under a temperature- and light- controlled environment and had free access to water and either standard chow or 55% fat (Harlan Teklad, Madison, WI, USA) rodent diets. The high fat diet contained 68% saturated fatty acids (oleic, linoleic and linolenic acid) and 32% unsaturated fatty acids (palimitic and stearic acid). Fasted animals were deprived of chow for 15- to 18-hr (overnight) before the experiment. The Stockholm North Animal Ethical Committee approved all protocols.

Anesthesia and Tissue Sampling

Rats undergoing surgeries for systemic infusion were anesthetized with 125 mg/100g body weight (bw) of ketamine. The day of the *in vitro* experiment animals were anesthetized with either 60 mg/kg bw of sodium pentobarbital (rat) or 0.02 ml/g bw of 2.5% Avertin (mouse).

In vivo glucose infusion

Rat hind-limb muscles were removed immediately after infusion and stored at -80^{o}C for later analysis.

In vitro muscle incubation

Extensor Digitorum Longus (EDL), epitrochlearis and soleus muscle were dissected from the animal, keeping the proximal and distal tendons intact for *in vitro* muscle incubation. Prior to incubation, the epitrochlearis muscle was placed on filter paper moistened with Krebs-Henseleit Buffer (KHB) and excess tissue was removed (Wallberg-Henriksson et al. 1987). Brain, diaphragm, gastrocnemius, heart, liver, quadriceps and white adipose tissue were frozen in liquid nitrogen immediately after isolation for biochemical analysis, determination of protein expression and activation. Blood sample were collected from the tail vein using heparinized microcapillaries (Drummond Scientific Co., USA), centrifuged for 20 min at 2,000 g for plasma sampling and stored at -80^{o}C for later analysis.

IN VIVO METHODS

Hyperglycemic Infusion

The *in vivo* effect of a 3-hr high glucose infusion on insulin signaling was assessed using a hyperglycemic infusion protocol (paper I). A catheter was placed in the jugular vein of GK and Wistar rats for systemic infusion. Animals were used for experiment 4 days after surgery and were studied under euglycemic (glucose levels at ~5 mM) or hyperglycemic (glucose levels maintained at ~20 mM) conditions, in the absence or presence of insulin. For all experimental conditions, endogenous insulin secretion was suppressed by a constant infusion of somatostatin (2 μg/kg.min). Sixty minutes after the onset of somatostatin infusion, animals received either saline (euglycemic) or a bolus of glucose (0.5 ml of 30% glucose), followed by continuous glucose infusion to raise blood glucose concentration to approximately 20 mM (hyperglycemic). Three hours thereafter, animals were either sacrificed or administered a bolus of insulin (20 mU, Actrapid, Novo Nordisk, Bagsvaerd, Denmark), followed by a continuous insulin infusion (3 U/min/kg bw) over a 20-min period. Blood samples for plasma glucose and insulin concentration were collected from the tail vein at different time points during the infusion.

Blood Chemistry

Blood glucose was measured using a One Touch glucose monitor (Lifescan Inc., Milpitas, CA, USA). Plasma insulin was measured using a Radioimmmunoassay (Rat Insulin RIA Kit, Lincoln Research Inc., USA) (paper I).

Tolerance Tests

Glucose (2 g/kg bw), insulin (0.75 mU/kg bw) or AICAR (0.25 g/kg bw) was injected intraperitoneally to overnight fasted animals. Blood was sampled from a tail incision before and during the glucose tolerance test. Blood glucose concentration was measured using a One Touch glucose monitor as described above (paper III and IV).

Swimming Exercise Protocol

The effect of exercise on skeletal muscle glycogen content was determined in paper IV. Age and weight matched AMPKγ3 transgenic and knock-out mice were assigned to sedentary or exercise groups. Six mice swam together in a plastic container (45 cm diameter) {Musi, 2001 #285}filled with water to a depth of 40 cm. Water temperature was maintained at 33-35^{o}C. Mice swam for 4 X 30-min intervals separated by 5-min rest periods. After the last swim interval, mice were dried and either studied immediately or put in their cage for a 2.5-hr recovery period. At the onset of the recovery period, mice received an intraperitoneal glucose injection as described above and were given free access to standard food and water.

IN VITRO METHODS

Muscle Incubation

Incubation media were prepared from a pre-gassed (95% O_2 /5% CO_2) stock of KHB containing 5 mM 4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid (HEPES) supplemented with either 0.1% Bovine Serum Albumine (BSA) (RIA grade) or 3.5% BSA Fatty Acid Free (FAF). Isolated muscles were incubated in either 1 ml (mice) or 2 ml (rats) of media in a shaking water bath. Water temperature was maintained at 30^{o}C. The gas phase in the vial was 95% O_2 /5% CO_2. Incubation media contained 5 mM glucose and either 15 mM (papers II, III, IV) or 35 mM (paper I) mannitol. Immediately after dissection and before stimulation, muscles recovered in oxygenated KHB for 15-30-min. Insulin-, AICAR- and PMA-stimulated conditions are explained in detail in each paper.

Hyperglycemic Stimulus

To study the direct effect of hyperglycemia on insulin signaling, the infusion protocol previously described in an *ex vivo* experimental setting was mimicked (paper I). After pre-incubation, skeletal muscles were incubated at 30^{o}C for 3-hr in KHB supplemented with 0.1% BSA and 25 mM glucose. To prevent a shortage of glucose in

the incubation media, muscles were transferred to identical fresh media after 1.5-hr. Thereafter muscles were incubated in the absence or presence of 60 nM insulin for 20-min.

Hypoxic Stimulus

Hypoxic condition is achieved by pre-gassing KHB with 95% N_2/5% CO_2. KHB is then supplemented with 0.1% BSA (RIA grade). Isolated rat muscles were incubated in hypoxic media for 80 min in a shaking water bath (paper III). Water temperature was maintained at 30^{o}C. The gas phase in the vial was 95% N_2/5% CO_2. Muscles were either frozen immediately for later analysis or transferred to oxygenated glucose free media containing 2 mM pyruvate for 15-min. Thereafter, glucose transport activity and AMPK and PI3K activation were measured.

Muscle Contraction Stimulus

The contraction protocol is designed so that muscle undergoes a tetanic isometric contraction event. Following pre-incubation, isolated mouse muscles were placed inside a temperature-controlled (30^{o}C) stimulation chamber and immersed in 4 ml of KHB identical to pre-incubation condition. Each muscle was positioned between two platinum electrodes with the distal tendon mounted to the bottom of the chamber. The proximal tendon was connected to a jeweler's chain, which was fixed to an isometric force transducer (Harvard Apparatus Inc., South Natick, MA, USA). Resting tension was adjusted to 0.25 g for mouse EDL (paper II) or 0.5 g for rat epitrochlearis muscle (paper III). Isometric tension development during the contraction protocol was recorded using a compact 2-Channel Student Oscillograph (Harvard Apparatus Inc.). Isometric muscle contraction was achieved via electrical stimulation. Muscles were stimulated at a frequency of 100 Hz (0.2 ms pulse duration, 10 V amplitude), delivered at a rate of 0.2-s contraction every 2-s for 10-min. Pulses were generated by a Tektronix TM 503 Power Module (Beaverton, OR, USA) and amplified on a 4-Channel Power Amplifier (Somedic, Sollentuna, Sweden). Basal muscles were treated as described above, minus electrical stimulation. Muscles were then either frozen immediately for biochemical analysis, protein expression and activation measurements or further incubated for the assessment of metabolic readouts.

Glucose Transport

After pre-incubation, muscles were incubated at 30^{o}C for 10-min in glucose-free KHB supplemented with 0.1% BSA and 20 mM mannitol to rinse glucose from the extracellular space (papers III, IV). The rate of glucose transport is linear between 5- and 120-min in rat epitrochlearis muscle (Hansen et al. 1995). Thereafter, glucose transport activity was determined using 2-deoxy-[1,2,^{3}H]-glucose (2.5 μCi/ml). Extracellular space was measured using [^{14}C]-mannitol (0.7 μCi/ml). Muscles samples were dissolved in 0.5 N NaOH and counted for [^{3}H] and [^{14}C] activity by liquid scintillation. Intracellular glucose accumulation was assessed by dual label calculation as described (Wallberg-Henriksson et al. 1987).

Oleate Oxidation

Muscles were pre-incubated at 30°C in the presence of insulin with or without AICAR for 20-min in KHB supplemented with 3.5% BSA-FAF and 10 mM glucose (paper IV). Thereafter, muscles were incubated in identical medium containing [1-^{14}C]-oleate (0.4 μCi/ml) for 1-hr. The medium was acidified by 0.5 ml of 15% Δ′-pyrroline-5-carboxylic acid, and liberated CO_2 was collected in center wells containing 0.2 ml of Protosol for 1-hr. Center wells were removed and counted for [^{14}C] activity by liquid scintillation.

Biochemical Analysis

Gastrocnemius muscle was removed from anesthetized animal as mentioned earlier. Glycogen was determined fluorometrically on muscle HCl extracts, as described previously (Lowery and Passonneau 1972). For TG measurements, skeletal muscle was homogenized in a heptan:isopropanol 3:2, Tween 1% mixture and removed of free glycerol. Thereafter, skeletal muscle TG were measured by a colorimetric assay with a TG/glycerol blanked kit (Roche Applied Science, Paris, France) using Seronorm™ lipid as standard.

Tissue Homogenization

Mouse or rat tissues were homogenized on ice in homogenization buffer, as described in each paper. Protein was determined on homogenates using a commercially available kit based on the Bradford method (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Protein and Lipid Kinase Activity Measurement

Following protein determination, aliquots of muscle or cell homogenates were immunoprecipitated with the appropriate antibody previously equilibrated with either protein A sepharose (PI3K assay), protein G sepharose (AMPK assay) or protein A/G sepharose mix (PDK-1 and PKC-ζ assay). Immunoprecipitates were washed in buffer, as described in each paper. Thereafter protein kinase activity was determined in the presence of the kinase substrate and coactivator when needed, and [γ-^{32}P]-ATP (table 1). For PI3K activity measurement, the radioactive lipid product was identified by thin layer chromatography and quantified by autoradiography. For protein kinase activity determination, the radioactive peptide product was spotted on phosphocellulose paper (P81) and radioactivity was assessed by liquid scintillation counting. Table 2 presents the characteristics of the antibodies used for immunoprecipitation.

Table 2. Protein Kinase Assay Reaction Conditions

PROTEIN KINASE		SUBSTRATE	Coactiv.	REACTION CONDITIONS	PRODUCT
AMPK	cell	LKKLTLRPSFSAQ (ADR1)	AMP	10 min, T_{room}	^{32}P-ADR1
	muscle	HMRSAMSGLHLVKRR	AMP	60 min, 30^{o}C	^{32}P-SAMS
PDK-1	muscle	KTFCGTPEYLAPEVRREPRILSEE EQEMFRDFDYIADWC (PDK-tide)	-	10 min, 30^{o}C	^{32}P-PDKtide
PI3K	muscle	L-α-phosphatidylinositol (PI)	-	20 min, T_{room}	PI-$^{32}P_3$
PKCζ	muscle	ERMRPRKRQGSVRRRV	PS	20 min, T_{room}	^{32}P-peptide

Western Blot Analysis

Following protein determination, aliquots of tissue homogenates were suspended in the Laemmli sample buffer (Laemmli 1970). Equal amounts of protein were separated by sodiumdodecylsulfate polyacrylamide gel electrophoresis and transferred to polyvinylidenedifluoride membranes. Thereafter the membrane was blocked with 7.5% non-fat milk for 2-3-hr at room temperature and incubated with primary antibody (table 2) overnight at 4 ^{o}C. The membrane was then washed with Tris-Buffered Saline Tween (TBST) (10 mM Tris, 140 mM NaCl, 0.02% Tween, pH 7.6) and incubated with appropriate secondary antibody for 1-hr at room temperature. After an additional washing with TBST, the proteins of interest were visualized by enhanced chemiluminescence (ECL or ECL+, Amersham Biosciences, Buckinghamshire, UK) and subsequent autoradiograph. The corresponding bands were quantified by densitometry (Molecular Analyst Software). Table 2 presents the characteristics of the antibodies used for Western Blot analysis.

RNA Extraction and cDNA Synthesis

Skeletal muscle RNA was extracted using Trizol reagent (Sigma) and purified with isopropanol and ethanol. Thereafter purified RNA was treated with DNAse I using DNA-free kit (Ambion, Huntingdon, UK) according to Ambion's protocol. DNAse-treated RNA then served as template for cDNA synthesis using SuperScript First Strand Synthesis System (Invitrogen, Stockholm, Sweden) and appropriate oligo(dT) primers.

Quantitative PCR

mRNA quantification was performed using real-time PCR with the ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Warrington, UK) and SYBR-green. The relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against housekeeping gene 36B4 (acidic ribosomal phosphoprotein). Primers were selected using PRIMER EXPRESS (Applied Biosystems). Transcript sequences were obtained from ENSEMBL and GENBANK databases.

Table 3. Antibody Characteristics

ENZYME		EPITOPE	MW (kDa)	SOURCE
ACC	P-ser 79	rat aa 73-85	257	Upst. Biotech., Lake Placid, NY
	ACCβ expr.	hum. aa 1501-1638; C-terminal	257-280	Upst. Biotech.
AMPK	P-thr 172	hum. α Synthetic Peptide (SP)	62	Cell Sign. Tech., Beverly, MA
	IP	rat α1 and α2	-	Gift from Arexis, Sweden
	anti-α1 expr.	hum. aa 376-392	63	Upst. Biotech.
ERK	P-tyr 204/ P-thr 202	hum. aa 196-209	44/42	New Engl. Biolab., Boston, MA
	expr.	rat aa 219-358	44/42	Transd. Lab., Lexington, KY
JNK	P-thr 183/ P-tyr 185	synth. phospho-peptide	46/54	Cell Sign. Tech.
	expr.	hum. JNK2	46/54	Cell Sign. Tech.
p38	P-thr 180/ P-tyr 182	rat SP	43	Cell Sign. Tech.
	expr.	hum. SP	43	NEB
PDK-1	P-thr 373/376	hum. SP	60-68	Cell Sign. Tech.
	P-ser 241	hum. SP	63	Cell Sign. Tech.
	expr.	hum. C-terminal	58-68	Cell Sign. Tech.
PKB	P-ser 473	mouse SP	62	NEB
	expr.	mouse SP	62	NEB
PKCα/β	P-thr 638/641	hum. α SP	82/80	Cell Sign. Tech.
	α expr.	hum. aa 270-427; ATP BD	82	BD Biosciences, CA
	β expr.	hum. aa 126-324;Ca++ BD	80	BD Biosciences
PKCδ	P-ser 643	human SP	78	Transd. Lab.
	expr.	hum. aa 114-289; Ca++ BD	78	Cell Sign. Tech.
PKCθ	P-thr 538	hum. SP	79	Cell Sign. Tech.
	expr.	hum. aa 21-217;Ca++ BD	79	Transd. Lab.
PKCζ	P-thr 403/410	hum. SP	76	Cell Sign. Tech.
	IP	rat C-terminal aa 577-592	-	Upst. Biotech.
	expr.	hum. PKCζ; substrate BD	72	Transd. Lab.
PI3K	P-tyr IP	hum. SP	-	Transd. Lab.

BD = binding domain;

SP = synthetic peptide designed to react with residues surrounding the sites of interest.

expr. = protein expression

Table 4. Primer Sequence

GENE	DATABASE ID	PRIMER	
		FORWARD	REVERSE
LPL	ENSMUST00000015715	CTGGGCTATGAGATCAACAAGGT	AGGGCATCTGAGAGCGAGTCT
CD36	ENSMUST00000003024	GCCAAGCTATTGCGACATGA	AAAAGAATCTCAATGTCCGAGACTTT
CPT1	ENSMUST00000023287	ACCACATCCGCCAAGCA	TTCCTCAGCTGTCTGTCTTGGA
UCP3	ENSMUST00000032958	GCCAGGGAGGAAGGAGTCA	TGACAATGGCATTTCTTGTGATG
Cit. Synth.	ENSMUST00000005826	GCAAATCAGGAGGTGCTTGTCT	TCTGACACGTCTTTGCCAACTT
Cytochr. C	NM007808	GGAAAAGGGAGGCAAGCATAA	GCAGCCTGGCCTGTCTTC
HAD	ENSMUST00000029610	TGGACGGGTGGCATGAA	TGGAAGGACTGGGCTGAAAT

Statistics

Data are represented as mean±SE. Differences between two groups were determined by unpaired Student's *t*-test. Differences between more than two groups were determined by one-way Analysis of Variance (ANOVA) followed by the Fisher's least significant difference *post hoc* analysis. Significance was accepted at $P<0.05$.

RESULTS and DISCUSSION

GK Rats are Hyperglycemic and Insulin Resistant

GK rats are a non-obese model of T2DM. They exhibit mild hyperglycemia (Song et al. 1999 and paper I) and glucose intolerance (Krook et al. 1997). In addition they display skeletal muscle insulin resistance at the level of glucose transport (Krook et al. 1997) and early and intermediate steps of the PI3K signaling cascade (Krook et al. 1997 and paper I). These defects are functional and fiber-type specific (Song et al. 1999).

GK rats are a useful model to study the toxic effects of glucose on muscle intracellular signaling. Indeed restoration of glycemia with phlorizin (Krook et al. 1997) or thiazolidinedione (Kanoh et al. 2001) normalizes signaling defects at intermediate steps of PI3K signaling pathway, suggesting that glycemia modulates insulin intracellular signaling. In paper I, the question of whether an acute elevation of glycemia would normalize or further impair insulin signaling to glucose transport in skeletal muscle from diabetic GK rats was addressed.

Acute Normalization of Glycemia Does Not Improve PI3K Signaling in GK Rats

Chronic hyperglycemia is a characteristic feature of insulin resistance and T2DM. Glucose itself can impair insulin-stimulated PI3K signaling, and this is referred to as "glucose toxicity". Mildly hyperglycemic GK rats exhibit impaired insulin action at the level of PI3K and PKB (Krook et al. 1997, Song et al. 1999 and paper I). These defects are functional, since protein expression is not affected (Krook et al. 1997, Song et al. 1999 and paper I). Normalization of glycemia results in normalization of insulin signaling at the level of PKB (Krook et al. 1997, Song et al. 1999, Oku et al. 2001), whereas the PI3K defect persists. Thus, PKB, rather than PI3K, appears to be sensitive to the level of glycemia. To test when hyperglycemia-induced defects at the level of PKB can occur acutely in skeletal muscle, Wistar rats were infused with 20 mM glucose for 3 hrs. Insulin-stimulated PI3K, PKB and ERK1/2 activation was unaltered by hyperglycemia under these conditions. Thus, a more prolonged period of hyperglycemia may be needed to impair PI3K signaling in normoglycemic rats rendered hyperglycemic. This hypothesis is consistent with *in vivo* observations, whereby hyperglycemic (streptozotocin-injected) 10 week-old rats exhibit altered insulin-stimulated PKB activity (Oku et al. 2001). In contrast, i*n vitro* observations provide evidence to suggest that 4 hrs seem to be sufficient to impair PKB activation (Kurowski et al. 1999). In both cases, appropriate PI3K action is retained.

We hypothesized that impaired insulin signaling in diabetic GK rats would be improved after acute normalization of glycemia, yet these signaling defects were evident, regardless of the level of glycemia (paper I). Interestingly, basal responses for PI3K and PKB were further reduced in GK rats, although protein expression was unaltered. Thus a more prolonged period of euglycemia in GK rats is also likely to be necessary to achieve improvements in insulin signaling. The mechanism by which hyperglycemia induces defects in PI3K signaling may involve intracellular accumulation of glucose metabolites, such as glucosamine (Parker et al., 2003),

impairments in serine kinases, or altered phosphatase activity (Filippis et al. 1997; Marques et al. 2000). Thus a 3-hr elevation of glycemia is insufficient to induce an impairment in insulin signaling at the level of PI3K and/or PKB.

Acute Hyperglycemia Differentially Modulates PKB and PKCζ

The possibility of a differential effect of glucose on parallel signal transduction pathways, such as PKB, PKCζ and ERK1/2, was evaluated. Basal PKCζ activity was elevated under hyperglycemic conditions in Wistar and GK rats, whereas ERK1/2 phosphorylation was unchanged in either animal model (paper I). To determine whether the glucose-induced effect on PKCζ is direct, isolated skeletal muscle was exposed to 5 or 25 mM glucose *in vitro*. Consistent with our *in vivo* observation, PKCζ activity and phosphorylation was increased in skeletal muscle exposed to 25 mM glucose (paper I). This observation is supported by earlier studies, whereby exposure of isolated skeletal muscle (Kawano et al. 1999) or mesangial cells (Kikkawa et al. 1994) to high extracellular concentrations of glucose was associated with PKCζ translocation. Interestingly normalization of glycemia in diabetic animals corrects defective PKCζ activation (Kanoh et al. 2000, Kanoh et al., 2001), suggesting that in addition to PKB, PKCζ is also modulated by the level of glycemia. Nevertheless PKCζ activation appears to be altered more rapidly than PKB.

The mechanism by which hyperglycemia induces PKCζ activation independently of PIP_3 is currently unknown, but might involve another acidic lipid, namely phosphatidic acid (PA). Indeed glucose has been reported to directly activate PKCζ through a sequential activation of the dantrolene-sensitive, non-receptor proline-rich tyrosine kinase-2 and phospholipase D (Bandyopadhyay et al. 2001). Activation of PLD results in intracellular accumulation of PA. Thus glucose may selectively activate PKCζ through a PI3K-independent mechanism involving PA.

Glucose Increases Conventional and Novel PKC Activity

To test whether glucose effects on PKCζ are isoform-specific, conventional and novel PKC activation was assessed after acute exposure of isolated skeletal muscle to 5 or 25 mM glucose *in vitro*. An increase in PKC-α/β and -δ but not -θ phosphorylation was noted (paper I). This is consistent with a previous study, whereby glucose was reported to activate several PKC isoforms including PKC-α, -δ, -ε and -ζ in Rat1 fibroblasts (Berti et al. 1994). PKC activation may account for glucose-induced insulin resistance (Kroder et al. 1996) through a glucosamine mediated mechanism (Filippis et al. 1997). Thus the acute effects of hyperglycemia on PKCζ are unspecific to this isoform.

Glucose Positively Modulates PDK-1

PKB, PKC and ERK are downstream substrates of PDK-1 and in that regard belong to the AGC kinases superfamily [comprising the prototypes protein kinase A (PKA), G (PKG), and C (PKC)]. To further delineate the differential effect of glucose on activation of these AGC kinases, glucose effects on upstream PDK-1 were determined. In isolated soleus muscle exposed to 25 mM glucose for 3 hrs, PDK-1 activity and tyrosine phosphorylation was increased 1.6-and 2-fold, respectively,

regardless of whether insulin was absent or present (paper I). Activation of PDK-1 by glucose provides a potential mechanism for the observed glucose-mediated changes in PKC activity. However glucose did not alter PKB or ERK1/2 phosphorylation indicating a divergence in the effects of PDK-1 on its downstream AGC substrates. Differences in PDK-1 intracellular localization following glucose exposure may provide a mechanism for the divergent effects on PKB and PKCζ. Indeed studies in 3T3L1 adipocytes overexpressing wild type or plasma membrane targeted PDK-1 show that full activation of PKB requires PDK-1 translocation to the plasma membrane, whereas PKCζ activation can be activated by cytosolic PDK-1 (Egawa et al. 2002).

Table 5. Glucose and insulin effects on PDK-1 activity and tyrosine and serine phosphorylation

PDK-1	activity	P-tyrosine	P-serine
insulin	↔	↑	shift
glucose	↑	↑	↑?
glucose + insulin	↑	additive	shift

Glucose-induced activation of PDK-1 appears to involve phosphorylation on tyrosine and serine residues, consistent with previously published observations (Casamayor et al. 1999; Park et al. 2001). In our experimental approach, insulin does not alter PDK-1 activity, despite increased tyrosine phosphorylation of the enzyme (table 5). Conversely, insulin was shown to induce a concomitant increase in tyrosine phosphorylation, activity and PDK-1 translocation to the plasma membrane in HEK293 cells transfected with PDK-1 (Park et al. 2001). Thus in our model, tyrosine phosphorylation of PDK-1 may be necessary, but insufficient for activation of the enzyme. In addition the observation that PDK-1 activation occurs independently of PIP_3 supports the dispensability of PIP_3-mediated plasma membrane relocalization.

Comparative Simultaneous Effect of Insulin on MAPK Signaling

In addition to PI3K signaling, insulin also turns on MAPK signaling. Thus, simultaneous activation of p38-MAPK, ERK1/2 and JNK by insulin was assessed by detailed time course and dose-response studies. Insulin induced a robust activation of all three MAPK in a similar time- and dose-dependent manner in soleus and EDL muscle. Half maximal phosphorylation was reached within 4 min, whereas maximal effects were noted after 20 min of insulin exposure. Moreover MAPK phosphorylation in response to insulin was maximal when insulin concentration reached 120 nM (paper II). A previous *in vivo* study provides evidence that the time course for insulin-induced activation of the three MAPK is distinctly different, with activation of JNK occurring within seconds, p38-MAPK within 2 min, and ERK1/2 occurring within 4 min (Moxham et al. 1996). Thus *in vitro* insulin activation of MAPK seems to take longer time than *in vivo*. This might be due to insulin-induced hemodynamic effects that render the hormone more effective in activating MAPK when in the systemic circulation. Nevertheless simultaneous *in vitro* activation of all three MAPK modules raises the question of specificity in signal transduction. MAPK are known to have both distinct and identical substrates (Chen et al. 2001), thereby

providing a mechanism for specific signaling as well as amplification of the response. This might be of particular importance in disease states.

One interesting observation is that insulin-stimulated ERK1/2 phosphorylation appears to be biphasic in EDL. This suggests that at least two distinct activating mechanisms may account for insulin-stimulated ERK1/2 phosphorylation. Recent evidence shows that the divergence occurs at the level of MEK, the upstream activator of ERK1/2. Indeed wortmanin -sensitive and -insensitive ERK1/2-activating pathways have been identified in a perfused rat hind limb model (Wojtaszewski et al. 1999). Wortmanin-insensitive activation of ERK1/2 signaling appears to be PKC-dependent (Nakamura et al. 1996), whereas wortmanin-sensitive ERK1/2 activation in muscle is PI3K-dependent. Thus insulin activation of ERK1/2 might recruit two distinct signaling mechanisms upstream of MEK mediated by PKC and PI3K, respectively and this might account for the observed biphasic-like insulin-stimulated ERK1/2 phosphorylation.

Fiber-Type Specific MAPK Signaling

Recent evidence shows that insulin activation of PI3K signaling is fiber-type specific (Song et al. 1999). To determine whether MAPK signaling also occurs in a fiber-type specific manner maximal (120 nM for 20 min) insulin effects on MAPK phosphorylation in soleus and EDL muscle were compared. Insulin action on p38-MAPK was more transient and less robust in oxidative versus glycolytic muscle, whereas ERK1/2 response to insulin was unchanged. Interestingly JNK protein expression was higher in glycolytic versus oxidative muscle, whereas p38-MAPK and ERK1/2 protein expression was similar (paper II). This observation is counter intuitive, since slow-twitch oxidative fibers are generally considered to be highly sensitive to stimulation by insulin. However contraction induced p38-MAPK activation is reportedly increased in fast-twitch muscle, whereas ERK1/2 activation is similar between slow- and fast-twitch muscle fibers (Wretman et al. 2000).

Effect of Different Stimuli on MAPK Signaling

MAPK can be activated by different stimuli including insulin, contraction and PMA, a DAG analog. MAPK activation appears to require activation of specific MEKK upstream kinases to ensure specificity of the signal (Chen et al. 2001). However upstream MEKK activating mechanisms are uncertain. In paper II, the additive effects of insulin, contraction and PMA on MAPK phosphorylation was determined. Insulin and PMA elicited an additive response on JNK and ERK1 phosphorylation, whereas insulin and contraction did not elicit additive effects on any of the MAPK studied. Interestingly the combined effect of insulin and contraction on p38-MAPK phosphorylation was blunted (paper II). This is consistent with previous reports of reduced insulin action on PI3K in exercised skeletal muscle (Goodyear et al. 1995, Wojtaszewski et al., 1997). Although the effect of prior insulin exposure on contraction-stimulated MAPK activation was not assessed, one could speculate that during the initial phases of skeletal muscle contraction, the energy supply for intracellular signaling is turned on, whereas processes involved in energy storage are turned off. Since insulin elicits a storing signal and the contracting muscle needs energy, the skeletal muscle may become temporarily insulin resistant.

Ob/ob mice are Insulin Resistant

Insulin resistance in *ob/ob* mice is rather well-defined and partly mirrors the defects described in obese diabetic patients. Impaired insulin signaling in this animal model is associated with reduced IR number and activation (Kahn et al. 1973). The effect of the remaining insulin signaling through IR on insulin-stimulated MAPK activation was determined in skeletal muscle from obese insulin resistant *ob/ob* mice. Insulin-stimulated ERK1/2 and JNK phosphorylation was reduced, whereas absolute p38-MAPK activation was unchanged. These defects are functional since ERK1/2 and JNK protein expression was intact (paper II). Insulin activation of ERK1/2 is also impaired in muscle from obese diabetic Zucker rats (Osman et al. 2001). This suggests that insulin activation of ERK1/2 and JNK may be mediated by IR/IRS-1 signaling. In contrast, p38-MAPK protein expression was reduced in parallel with increased basal phosphorylation, consistent with a previous observation in skeletal muscle from T2DM patients (Koistinen et al. 2003). Furthermore basal ERK1 in EDL (paper II) and JNK phosphorylation in soleus (paper II) tended to be elevated (non significant), consistent with observations in adipocytes from T2DM subjects (Carlson et al. 2003). Normalization of basal p38-MAPK phosphorylation prevented the insulin-stimulated decrease in GLUT4 protein levels (Carlson et al. 2003), suggesting that aberrant p38-MAPK activation contributes to the pathogenesis of insulin resistance. Thus insulin-stimulated activation of all three MAPK is impaired in muscle from obese insulin resistant mice, whereas basal p38-MAPK phosphorylation is increased.

Contraction Effects on ERK1/2 and JNK Activation is Intact in *ob/ob* Mice

Defects in insulin-stimulated PI3K (table 6) and MAPK signaling (paper II) contribute to skeletal muscle insulin resistance in *ob/ob* mice. Moreover, contraction- and hypoxia-stimulated glucose transport is preserved in insulin resistant obese *fa/fa* rat (paper III). To determine whether insulin-independent MAPK activation is altered or preserved in insulin resistant *ob/ob* mice, isolated EDL muscle was contracted and/or incubated with PMA. Contraction elicited a robust activation of all three MAPK in lean mice. In addition contraction-stimulated ERK1/2 and JNK phosphorylation was intact in EDL muscle from *ob/ob* mice, whereas p38-MAPK phosphorylation was slightly reduced (non significant) (paper II). This might be due to reduced p38-MAPK protein expression. MAPK have been postulated to mediate exercise-induced improvement in insulin sensitivity, although the underlying mechanism remains largely uncertain. There is evidence supporting that p38-MAPK might directly intervene at the level of GLUT4 activation (Somwar et al. 2001), whereas ERK1/2 appears to be dispensable for exercise-stimulated glucose transport (Wojtaszewski et al. 1999). Thus activation of ERK1/2 and JNK via insulin-independent stimuli such as contraction may bypass defective insulin signaling in insulin resistant obese states.

PMA Effects on ERK1/2 and JNK Activation is Intact in *ob/ob* Mice

PKC isoforms have been suggested to be upstream activators of MAPK following insulin and contraction stimulation (Letiges et al. 2002, Formisano et al., 2000, Chen et al., 2002). Activation of conventional and novel PKC isoforms can be induced by the use of PMA. The effects of PMA on MAPK phosphorylation in *ob/ob*

mouse skeletal muscle were determined. PMA elicited a robust activation of all three MAPK in lean mice. This is consistent with the literature whereby PKC are shown to be upstream activators of all three MAPK (Tsakiridis et al. 2001). In *ob/ob* mice, PMA-induced ERK1/2 phosphorylation was preserved, whereas effects on JNK tended to be reduced (~29%, non significant) (paper II). This divergence in ERK1/2 and JNK phosphorylation may be due to PKC isoform specific activation, since ERK1/2 and JNK protein expression was unaltered. This is consistent with PKC-ε isoform-specific MAPK activation in heart (Baines et al., 2002). Altered PKC-α, -ε and -θ isoform distribution in skeletal muscle is also associated with an insulin resistance phenotype in Zucker *fa/fa* rats (table 6). Another possibility is that PKC have positive effects on ERK1/2 and negative effects on JNK activation, as shown in rat adipocytes and L6 myotubes incubated in the presence of a PKC inhibitor (Standaert et al. 1999). PMA induced p38-MAPK phosphorylation was totally blunted in *ob/ob* mice in response to PMA, possibly due to the reduction in protein expression (paper II). However the results obtained for contraction-stimulated p38-MAPK phosphorylation suggest that reduced protein expression only partially accounts for blunted PMA effects on p38-MAPK activation and additional upstream regulatory mechanisms might be impaired. This is the first evidence showing that ERK1/2 and JNK signaling are intact following insulin-independent PMA stimulation of EDL muscle from insulin resistant obese mouse model. However caution should be made regarding the role of PKC isoforms, since specific positive and negative involvement of these kinases can play a role in the development of insulin resistance (Kim et al. 2004, Li et al., 2004).

Zucker *fa/fa* Rat are Glucose Intolerant and Insulin Resistant

Rats homozygous for the leptin receptor (*fa*) gene develop severe obesity and a T2DM-like syndrome. The absence of leptin receptor leads to dysregulation of central energy balance, resulting in hyperphagia and subsequent profound central and visceral obesity (Yamashita et al. 1997; Crouse et al. 1998; da Silva et al. 1998). Whole body lipid oversupply causes glucose intolerance and skeletal muscle insulin resistance at the level of glucose transport and PI3K signaling (paper III).

Exercise is an alternate insulin-independent pathway to increase glucose transport and counteract muscle insulin resistance. AMPK is a key mediator of exercise-stimulated glucose transport in skeletal muscle and therefore a potential therapeutic target to combat insulin resistance in T2DM. Isoform specific activation of AMPK was determined in skeletal muscle from obese insulin resistant Zucker *fa/fa* rats to assess whether this pathway is impaired or preserved.

Contraction-Stimulated AMPKα1 Isoform Specific Defect in *fa/fa* Rat

T2DM and obesity are associated with skeletal muscle insulin resistance at the level of glucose transport (Zierath et al. 1996) and fatty acid oxidation (Kelley et al. 1994). AMPK regulates glucose and lipid metabolism (Carling 2004), by an insulin-independent mechanism. Skeletal muscle expresses AMPK-α1 and -α2 catalytic subunits (Stapleton et al. 1996). Thus contraction- and hypoxia-stimulated AMPKα catalytic subunit specific activation was measured in glycolytic muscle of obese insulin resistant Zucker *fa/fa* rats (paper III). Hypoxia and contraction increased AMPK-α1 and -α2 activity in lean rats, consistent with previous observations (Mu et al. 2001).

Moreover in Zucker *fa/fa* rats, a contraction-stimulated AMPKα1-isoform specific defect in glycolytic muscle was observed. In contrast, hypoxia-stimulated AMPKα1 activation was normal. Thus contraction and hypoxia differentially modulate AMPKα1-subunit activation in glycolytic *fa/fa* muscle. Furthermore hypoxia had a greater effect on AMPKα1 activity compared to contraction suggesting that the intensity of the cellular distress determines AMPKα isoform-specific activation. This is consistent with AMPKα1-subunit being activated by high intensity exercise (Fujii et al. 2000) and might be due to the ATP-depleting effect of hypoxia that is more dramatic than the contraction effect (Fujii et al. 2000). In Zucker *fa/fa* rat (Bergeron et al. 2001) and T2DM patients (Musi et al. 2001), AMPKα-subunit protein expression is unaltered in skeletal muscle.

Basal AMPKα1-associated activity was reduced in skeletal muscle from Zucker *fa/fa* rats (paper III). This observation is consistent with previous observations in T2DM patients, whereby AMPKα1 activity tended to be decreased (Musi et al. 2001). AMPK is a stress sensor and stress factors inherent to these experiments – i.e. muscle stretch (Wojtaszewski et al. 2000) - might have revealed an AMPKα1 isoform-specific defect in glycolytic *fa/fa* muscle. In addition nutrient oversupply in Zucker *fa/fa* rat might differentially regulate AMPKα-subunit specific function (Musi et al. 2001). Since several AMPK upstream kinases are likely, an AMPKα1 upstream kinase-specific defect in glycolytic muscle of obese insulin resistant Zucker *fa/fa* rat cannot be excluded.

AMPKα2 activation was normal under both contraction- and hypoxic-stimulatory conditions (paper III). Furthermore AMPK-α1 and -α2 isoforms appear to be regulated independently by different stimuli. Since activation of α2, but not α1, is associated with a decrease in phosphocreatine:creatine (P-Cr:Cr) ratio (Fujii et al. 2000), contraction and hypoxia might induce distinct changes in AMP:ATP and P-Cr:Cr ratios that differentially activate AMPK. Thus the intensity of the stimuli and the resulting distinct intracellular stress may determine AMPKα isoform-specific activation. This is consistent with AMPKγ-subunits differing in their AMP sensitivity and muscle fiber type specific AMPK heterotrimer expression (Mahlapuu et al. 2004). Furthermore AMPKα catalytic subunit substrate specificity (Woods et al. 1996) and isoform specific subcellular localization (Salt et al. 1998) may provide additional regulatory mechanisms for AMPK isoform specific activation by distinct stimuli.

Contraction- and Hypoxia-Stimulated Glucose Transport is Intact in *fa/fa* Rats

Contraction- and hypoxia-stimulated glucose uptake was intact in glycolytic muscle from obese Zucker *fa/fa* rats despite insulin resistance (paper III), consistent with previously published observations (Azevedo et al. 1995). Thus the glucose transport effector system is intact in diabetic muscle stimulated with insulin-independent stimuli. Substantial evidence suggests that AMPK is activated during contraction and hypoxia, concurrently with an increase in glucose uptake paper III (Bergeron et al. 1999, Hayashi et al. 2000, Hayashi et al. 1998, Mu et al. 2001). Thus AMPK activation may prove a valuable strategy to bypass obesity-driven skeletal muscle insulin resistance through activation of the glucose transport effector system.

Contraction-stimulated glucose transport is preserved in glycolytic muscle from Zucker *fa/fa* rat despite impaired contraction-stimulated AMPKα1 activity. This suggests that either AMPKα1-aubunit is dispensable for contraction-stimulated glucose

uptake or the remaining AMPKα1 activity is sufficient to transduce contraction-stimulated glucose transport. AMPKα2 appears to be the major isoform recruited for contraction- (Vavvas et al. 1997) or exercise- (Musi et al. 2001) stimulated glucose transport. In contrast, in knock out mice, α1 but not α2 mediates contraction-induced glucose uptake (Jorgensen et al. 2004). The identity of the catalytic subunit that specifically participates in contraction-stimulated glucose transport is unclear. The intensity of the contraction protocol may determine AMPK isoform-specific recruitment. Indeed moderate exercise preferentially activates AMPK-α2 whereas high intensity exercise selectively activates AMPK-α1 subunit (Fujii et al. 2000, Wojtaszewski et al. 2000). Not surprisingly skeletal muscle glycogen content and fiber type also play a role (Derave et al. 2000). Finally AMPK might not be the sole mediator of contraction-stimulated glucose transport in skeletal muscle (Jorgensen et al. 2004, Mu et al. 2001, Musi et al. 2001).

Table 6. Signaling defects in muscle from animal models of insulin resistance

Insulin signaling defects in PI3K and MAPK cascades account for insulin resistance in animal models of T2DM and obesity. Understanding the metabolic cause of the defects, as well as insulin-independent AMPK and MAPK activation, may prove a resourceful alternative to treat insulin resistance.

(B=basal, I=insulin, P=PMA, C=contraction stimuli; n.d.=not determined. ↑=increased, ↓=decreased, ↔=unchanged compared to insulin-stimulated conditions)

			HIGH FAT	GK	*ob/ob*	*fa/fa*
PI 3-K	**IR**	activ.	↔ (8) ↓ (16)	↓ (5)	↓ (7)	↓ (1)
		expr.	↔ (8)	↔ (21)	↓ (7)	↓ (1)
	IRS-1	activ.	↓ (19) ↔ (23)	↓ (21)	↓ (7)	↓ (1)
		ser-P	↑ (19)	↔ (20)	↑ (6)	↑ (6)
		expr.	↔ (19)	↔ (21)	↓ (7, 18)	↓ (1)
	PI 3-K	activ.	↓ (22,24)	↓ (12, 21)	↓ (7,9,22)	↓ (2)
		expr.	↔ (24)	↔ (21)	↓ (7)	↓ (1)
	PKB	activ.	↓ (22,23)	↓ (15) ↔ (12)	↓ (22)	↓ (14)
		expr.	↔ (22)	↔ (15)	↔ (22)	
	c,nPKC	activ.	ε, δ, θ ↑ (19) ↔ (19)	↑ (3) θ ↓ (3)	n.d.	↑α,ε,θ (3) δ ↓ (3)
		expr.		↔ (3) θ ↓ (3)	n.d.	↔ (3) δ ↓ (3)
	aPKCζ	activ.	↓ (22,23)	↔ (3) ↓ (13)	↓ (22)	↔ (3) ↓ (22)
		expr.	↔ (22)	↔ (3)	↔ (22)	↔ (3)
MAPK	**p38**	activ.	n.d.	n.d.	↑B ↓I,P,C (paperII)	↔ (10)
		expr.	n.d.	n.d.	↓ (paperII)	↔ (10)
	ERK-1/2	activ.	n.d.	n.d.	↔B ↓I ↔P,C (paperII)	↔ (10) ↓ (17)
		expr.	n.d.	n.d.	↔ (paperII)	↔ (11)
	JNK	activ.	n.d.	n.d.	↓I,P ↔C (paperII)	n.d.
		expr.	n.d.	n.d.	↔ (paperII)	n.d.
AMPK	**AMPK**	activ.	n.d.	n.d.	n.d.	↔ (4) ↓α1 ↔α2
		expr.	n.d.	n.d.	n.d.	↔ (4)

[1] Anai M. (1998) *Diabetes* **47**, 13-23 ; [2] Anai M. (1999) *Diabetes* **48**, 158-169 ; [3] Avigon A. (1996) *Diabetes* **45**, 1396-1404 ; [4] Bergeron R (2001) *Diabetes* **50**, 1076-1082 ; [5] Dadke SS. (2000) *Biochem. Biophys. Res. Commun*. **274**, 583-589 ; [6] Eckel J. (1985) *Endocrinology* **116**, 1529-1534 ; [7] Folli F. (1993) *J. Clin. Invest*. **92**, 1787-1794 ; [8] Hansen PA. (1998) *J. Biol. Chem*. **273**, 26157-26163 ; [9] Heydrick SJ (1993) *J. Clin. Invest*. **91**, 1358-1366 ; [10] Ivy J. (2004) *Med. Sci. Sports*, 1207-1211 ; [11] Jiang ZY. (1999) *Diabetes* **48**, 1120-1130 ; [12] Kanoh Y. (2001) *Endocrinology* **142**, 1595-1605 ; [13] Kanoh Y. (2000) *J. Biol. Chem.* **275**, 16690-16696 ; [14] Kim YB. (2000) *Diabetes* **49**, 847-856 ; [15] Krook A. (1997) *Diabetes* **46**, 2110-2114 ; [16] Nagy LJ (1990) *Acta Endocrinol Copenh* **122**:361-368; [17] Osman AA. (2001) *J. Appl. Physiol.* **90**, 454-460 ; [18] Saad MJA. (1992) *J. Clin. Invest*. **90**, 1839-1849 ; [19] Schmitz-Peiffer C. (1997) *Diabetes* **46**, 169-178 ; [20] Schmitz-Peiffer C. (2000) *Cell. Sign.* **12**, 583-594 ; [21] Song XM. (1999) *Diabetes* **48**, 1-7; [22] Standaert ML. (2004) *J. Biol. Chem.* **279**, 24929-24934 ; [23] Tremblay F (2001) *Diabetes* **50**, 1901-1910; [24] Zierath JR. (1997) *Diabetes* **46**, 215-233

Insulin signaling defects in PI3K and MAPK pathways account for skeletal muscle insulin resistance in animal models. This may be due to acute and/or chronic exposure to hyperglycemia, hyperinsulinemia, and/or hyperlipidemia. Defects in AMPKα1 activation do not alter either contraction- or hypoxia-stimulated glucose uptake in

skeletal muscle from Zucker *fa/fa* rat. In addition AMPKα2 activation is intact in response to hypoxia and contraction in an insulin resistant obese animal model. Thus AMPK activation by insulin-independent pathways appears to bypass impaired insulin signaling to glucose transport in insulin resistant states. Since AMPKγ3 is expressed specifically in glycolytic skeletal muscle, this isoform may offer a therapeutic opportunity for the treatment of skeletal muscle insulin resistance. The AMPKγ3-subunit displays a naturally occurring missense mutation replacing arginine residue 225 by glutamine (R225Q) that renders the enzyme constitutively active. Functional analysis, as well as biological validation of the mutation is of crucial importance to ascertain AMPKγ3 as a putative target for the treatment of skeletal muscle insulin resistance. Therefore transgenic mice specifically overexpressing wild type (Tg-Prkag3^{wt}) or R225Q mutated (Tg-Prkag3^{225Q}) forms of AMPKγ3 in white muscle, as well as AMPKγ3 knock out mice (Prkag3$^{-/-}$) were generated using the classical targeted mutagenesis technology, and the role of AMPKγ3 in regulating whole body glucose homeostasis was determined.

The AMPKγ3-R225Q Mutation Leads to a Constitutively Active and Simultaneous AMP-Insensitive Kinase

AMPK is allosterically activated by AMP and covalently phosphorylated by AMPKK (Hardie et al. 1997). Thus two levels of intervention modulate AMPK activation and this dual regulation renders assessment of AMPK activation *in vitro* challenging. In paper IV, the effect of the R225Q mutation on AMPK activity and phosphorylation by upstream AMPKK was determined in Tg-Prkag3^{wt}, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice following insulin-independent stimulation. Basal-, AICAR- and contraction-stimulated AMPK phosphorylation was similar between genotypes. However phosphorylation of the AMPK target Acetyl CoA Carboxylase (ACC) was elevated under basal conditions and after AICAR stimulation in Tg-Prkag3^{225Q} mice. Thus the R225Q mutation appears to induce constitutive activation of AMPK. This contrasts observations in mutated pigs harboring the naturally occurring mutation, whereby AMPK activity in crude muscle membranes was found to be diminished (Milan et al. 2000). Thus the initial report suggested that the R225Q mutation might be a loss of function mutation. However identical mutations in Prkag1 (Hamilton et al. 2001) and Prkag2 (Arad et al. 2002) also result in constitutive activation of AMPK with a concomitant loss of AMP regulation.

The R225Q mutation is a gain of function mutation

Chronic activation of AMPK *in vivo* is correlated with an increase in glycogen content in skeletal muscle (Holmes et al. 1999). A glycogen supercompensation phenotype was observed in mutant pigs (Milan et al. 2000) and confirmed in Tg-Prkag3^{225Q} mice (paper IV). Thus, the R225Q mutation is not a loss of function mutation. The reduced AMPK activity observed in mutant pigs might be due to inhibitory effects of high glycogen on AMPK activation. AMPKβ subunits appear to have a glycogen binding domain and may therefore respond to glycogen feedback mechanisms. Consistent with this is the increased basal phosphorylation on Thr^{172} observed in AMPKα2 dominant negative mice (Mu et al. 2001) that might result from

a negative feedback signal that tries to overcome or compensate for the inhibited AMPK activity.

The R225Q mutation leads to AMP insensitivity

To determine whether the R225Q mutation results in loss of AMP binding, AMPK activation was measured in COS cells expressing heterotrimers containing α2, β2 and γ3 wild type or R225Q mutated subunit. AMPK activity was increased 2-fold in the absence of AMP and not further increased in the presence of increasing concentrations of AMP. Thus the R225Q mutation in γ3 similarly to the R70Q mutation in γ1 and the R302Q in γ2, leads to AMPK constitutive activation and loss of AMP dependency (Adams et al. 2004). Consistent replacement of an arginine residue by a glutamine in the AMP-binding Cysthationine Beta Synthase-1 (CBS1) domain of AMPKγ-subunits might lead to conformational change and subsequent loss of AMP binding. Whether or not direct binding of AMP molecules is altered in the AMPKγ mutants is unclear (Adams et al. 2004).

AMPK and ACC activation are dissociated

Loss of AMP dependency should translate into loss of AICAR dependency. However, ACC phosphorylation was further increased in Tg-Prkag3^{225Q} mice. This might be an indirect effect of the mutation caused by an altered metabolic state (Rubink et al. 2004). In human muscle prolonged exercise results in marked AMPK phosphorylation that is not sufficient to maintain an elevated ACC phosphorylation (Wojtaszewski et al. 2002) suggesting that AMPK and ACC activation can be dissociated. Alternatively, the AMPKγ3-subunit may not directly mediate ACC phosphorylation, as suggested by unaltered phosphorylation in Prkag3$^{-/-}$ mice and unaltered fatty acid oxidation in Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. To date whether γ1 or γ2 is necessary to mediate AMPK-induced ACC phosphorylation remains undefined.

AMPKγ3-R225Q subunit replaces endogenous γ3

The finding of increased AMPK phosphorylation in COS cells transfected with mutant α2β2γ3 heterotrimer does not translate to the *in vivo* situation. To determine whether there is a dilution effect by endogenous γ3 or γ1 isoforms, relative mRNA and protein expression was measured in glycolytic and oxidative skeletal muscle tissues of wild type, Tg-Prkag3^{wt}, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. Endogenous γ3 tended to decrease in glycolytic (EDL, gastrocnemius and quadriceps) muscle from either Tg-Prkag3^{wt} or Tg-Prkag3^{225Q} mice, whereas expression in oxidative soleus muscle was low and unchanged. In addition γ1 mRNA expression was reduced in glycolytic gastrocnemius and quadriceps of either Tg-Prkag3^{wt} or Tg-Prkag3^{225Q} mice, whereas levels in EDL and soleus remain unaltered. Thus the γ3 mutant isoform presumably replaces endogenous γ3 in transgenic mice overexpressing wild type and mutant γ3 isoform. Protein expression analysis reveals that overexpression of the wild type transgene led to an increase in the amount of γ3 protein and a concomitant increase in α1, α2, and β2 subunits. Conversely, protein expression of the mutant form is challenging to assess by qualitative Western Blot analysis because endogenous and exogenous mutant forms of γ3 differ by a single amino acid substitution. However the

mutation is fully dominant and there is no significant difference in glycogen content between pigs expressing 50% or 100% of the mutant form (Milan et al. 2000). Thus AMPK expression in Tg-Prkag3^{225Q} resembles the expression pattern in wild type mice.

The AMPKγ3-R225Q Mutation Increases Skeletal Muscle Glycogen Content

To determine whether the AMPKγ3-R225Q mutation leads to glycogen supercompensation in mouse glycolytic muscle, glycogen content was measured in white gastrocnemius from wild type, Tg-Prkag3^{wt}, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. Glycogen content was similar in wild type, Tg-Prkag3^{wt} and Prkag3$^{-/-}$ mice, independently of gender. In contrast Tg-Prkag3^{225Q} mice exhibit a 2-fold increase in skeletal muscle glycogen content in both male and female mice (figure 6). This confirms the phenotype observed in pigs (Estrade et al. 1993) and establishes a causal relationship between the AMPKγ3-R225Q mutation and skeletal muscle glycogen accumulation. Human subjects harboring the AMPKγ2-R302Q mutation also display increased muscle glycogen storage (Arad et al. 2002) that causes the Wolff-Parkinson-White cardiomyopathy syndrome (Gollob et al. 2001). Thus AMPKγ-subunits play a key role in regulating intramuscular glycogen metabolism. The underlying mechanism is undefined. Current investigations suggest that AMPK might be a GS kinase, thereby modulating glycogen accumulation (Wojtaszewski et al. 2003).

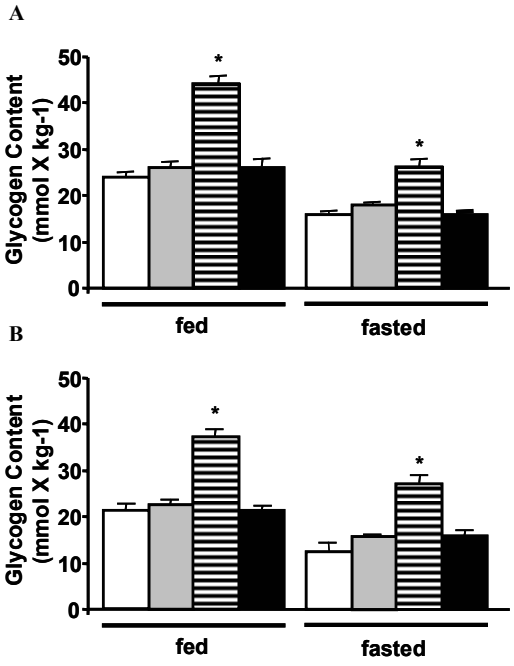
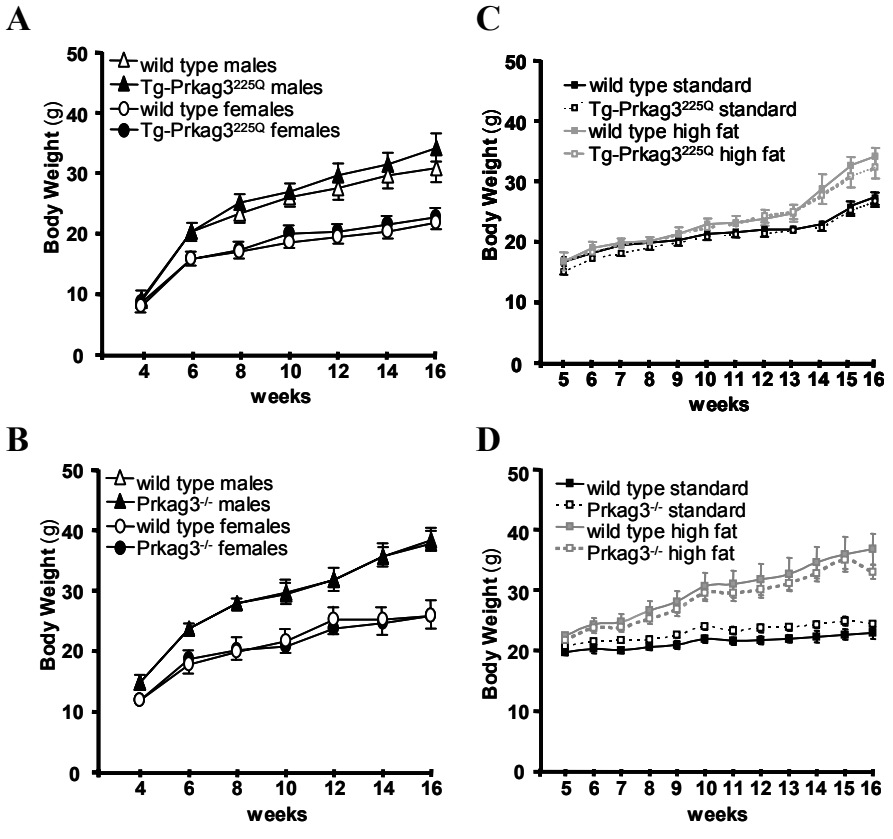


Figure 6. Tg-Prkag3 mice have elevated skeletal muscle glycogen content. Skeletal muscle glycogen content was assessed in fed and fasted male (A) and female (B) mice. Gastrocnemius muscle was excised from wild type (open bar), transgenic wild-type (gray-filled bar), Tg-Prkag3^{225Q} (horizontal lines) and Prkag3$^{-/-}$ (black-filled bar) mice. Glycogen content was assessed fluorometrically. Results are mean±SE for 5-30 mice. $P<0.01$ vs. wild type in the same condition.

Adaptation to chronic exercise is associated with increased glycogen storage (Greiwe et al. 1999). We (paper IV) and others (Andersson 2003) observed that AMPKγ3 controls the rate of glycogen resynthesis during recovery from exercise. In addition AMPK kinase dead mice have reduced skeletal muscle glycogen content and glycogen resynthesis 3-hr post-exercise (Mu et al. 2003), suggesting that AMPK activation is a determining factor in this process.

Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ Mice Have Normal Growth Rates

Since the AMPKγ3-R225Q mutation is activating (Andersson 2003), we hypothesized that Tg-Prkag3^{225Q} mice would be protected against diet-induced increase in body weight and adipose tissue accumulation, whereas insulin resistance in the Prkag3$^{-/-}$ mice would be more severe. In paper IV body weight and intraperitoneal adiposity was measured in wild type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice for 16 weeks.



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abdominal fat	wild type	Tg-Prkag3^{225Q}	Prkag3$^{-/-}$
standard diet	0.26±0.02	0.32±0.03	0.35±0.03
high fat diet	1.26±0.08	1.17±0.10	1.76±0.20*

Figure 7. Growth rates are normal in standard and high fat-fed Tg-Prkag3^{225Q} and Prkag3 $^{-/-}$ mice. Body weight in male and female Tg-Prkag3^{225Q} (A) and Prkag3$^{-/-}$ mice (B) and standard- (black lines) and high fat- (gray lines) fed Tg-Prkag3^{225Q} (C) and Prkag3$^{-/-}$ mice (D). Results are mean±SE for n=8-30 observations. P<0.05 vs. wild type in the same condition.

The R225Q mutation was without effect on body weight and adiposity in female mice, whereas a non significant trend towards increased body weight was observed in male mice (figure 7). Ablation of AMPKγ3 in Prkag3$^{-/-}$ mice was without effect on growth rates in either gender. Body weight was not measured in Hampshire pigs harboring the R225Q mutation. However increased lean body mass and reduced muscle water content was observed (Milan et al. 2000).

High fat feeding increases body weight and fat accumulation in wild type mice. Body weight is unaltered by either the R225Q mutation or ablation of AMPKγ3. In contrast intraperitoneal fat accumulation tends to be reduced in Tg-Prkag3^{225Q} mice, whereas Prkag3$^{-/-}$ display a trend for increased intraperitoneal fat mass (figure 7) already under standard diet (non significant). These observations suggest that AMPKγ3 isoform has a specific role in regulating lipid metabolism, a finding consistent with previously published observations showing that AMPKα2 knock out mice have increased fat accumulation because of increased TG accumulation in preexisting adipocytes (Villena et al. 2004) despite normal food intake (Viollet et al. 2003).

Glucose-, Insulin- and AICAR-Tolerance are Normal in Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ Mice

The effect of γ3 ablation and mutant γ3 overexpression in glycolytic muscle on whole body glucose homeostasis was determined. Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ display normal glucose and insulin tolerance (figure 8A and 8B), consistent with normal glucose tolerance noted in pigs carrying the R225Q mutation (B. Essén-Gustavsson, M. Jensen-Waern, R. Jonasson and L. Andersson, unpublished data). This suggests that the metabolic role of γ3 is restricted to skeletal muscle, consistent with γ3 isoform-specific expression in glycolytic muscle (Mahlapuu et al. 2004).

Treatment of diabetic animals with AICAR improves whole body glucose homeostasis (Song et al. 2002) presumably via AMPK activation. We hypothesized that constitutive activation of AMPK in Tg-Prkag3^{225Q} mice leads to enhanced AICAR sensitivity, whereas ablation of γ3 results in AICAR resistance in skeletal muscle. An AICAR tolerance test was performed in wild type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. AICAR tolerance was normal in all mouse models (figure 8C). This finding was unexpected since these animal models display impaired AICAR-stimulated glucose transport (paper IV). The preserved AICAR tolerance might result from AICAR insensitivity in Tg-Prkag3^{225Q} and/or γ3-independent AICAR effects on fatty acid oxidation. Alternatively, there may be unspecific effects of AICAR on metabolism that override any AMPK-mediated response. The action of AICAR on metabolism is incompletely known and furthermore not restricted to muscle tissue.

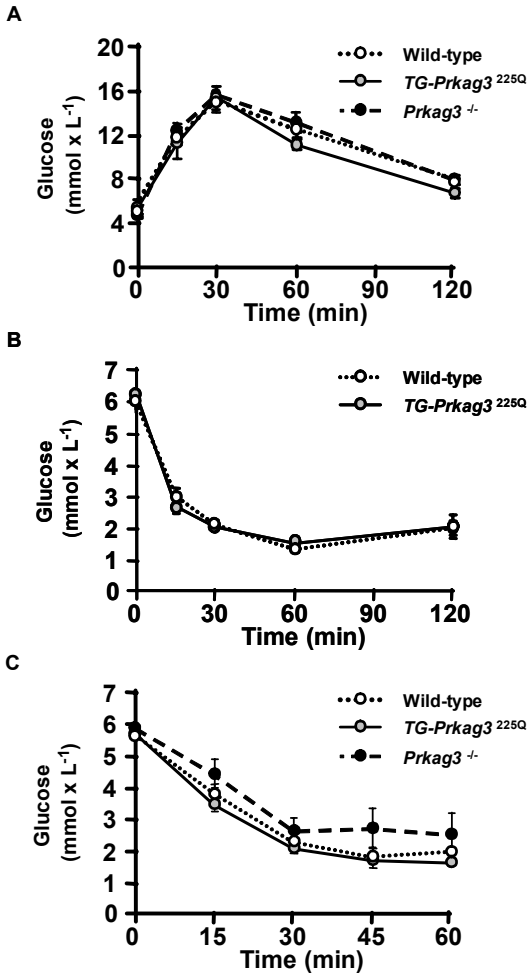

Figure 8. Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice have normal glucose, insulin and AICAR tolerance. Glucose (A, 2 g/kg bw), insulin (B, 0.75 mU/kg bw) and AICAR (C, 0.25 g/kg bw) tolerance was assessed in wild type (open circles/dotted line), Tg-Prkag3^{225Q} (gray circles/solid line), and Prkag3$^{-/-}$ (filled circles/dashed line) mice. Glucose tolerance was measured in fasted mice (n=10-15) whereas insulin and AICAR tolerance were measured in fed animals (n=5-8) after an intraperitoneal injection. Thereafter blood glucose was measured at different time points. Insulin tolerance was not assessed in Prkag3$^{-/-}$ mice. Results are mean±SE.

Nutritional Status Controls Insulin Sensitivity inTg-Prkag3^{225Q} Mice

Although AMPK is not directly regulated by insulin (paper III), it can modulate insulin signaling to glucose transport (Jakobsen et al. 2001). However results showing that AMPK activation leads to increased insulin sensitivity are based on short time (hours or days) activation of the enzyme, whereas the consequence of life long activation, as in genetically engineered mice is unknown. In paper IV, insulin-stimulated glucose transport was determined in mice overexpressing a constitutively active form of AMPKγ3 in glycolytic skeletal muscle. Tg-Prkag3^{225Q} mice are insulin resistant whereas Prkag3$^{-/-}$ display normal insulin responsiveness (figure 9B). Thus the AMPKγ3-R225Q mutation can induce skeletal muscle insulin resistance. This might be due to constitutive activation of AMPK on a life-long basis and sustained energy sensing. Consistent with this, insulin resistance was also observed at the level of fatty acid oxidation in Tg-Prkag3^{225Q} mice (figure 10).

Insulin and AMPK have opposing effects on intracellular metabolism in skeletal muscle. Insulin stimulates energy storage, whereas AMPK activation results in increased fuel utilization. Studies aimed to determine the effect of prior insulin or AICAR stimulation on AMPK activation and fuel metabolism reveal prior AMPK activation abolishes insulin's inhibitory effect on fatty acid oxidation (Winder et al. 2000). In addition, contraction markedly blunts insulin-stimulated activation of IRS-1

and PI3K (Goodyear et al. 1995). Thus AMPK activation might lead to insulin resistance. The underlying mechanism might be negative feedback from accumulated intracellular metabolites such as glycogen or lipids. Indeed patients with Mac Ardle's disease (i.e. patients with chronic muscle high glycogen content) exhibit decreased skeletal muscle insulin action (Nielsen et al. 2002). This is consistent with the observation that insulin resistance in Tg-Prkag3^{225Q} mice is lost with fasting (figure 9A) and that the insulin response is unaltered in Prkag3$^{-/-}$ mice. Although the R225Q mutation results in loss of AMP dependency, one can not exclude the possibility that the other intact CBS domains are still AMP sensitive and this may account for a small increase in AMPK activity and subsequent insulin resistance. Thus nutritional status determines insulin resistance in Tg-Prkag3^{225Q} mice and this corresponds to an increased flexibility in signaling that couples metabolic flexibility.

The AMPKγ3-Subunit is Necessary for AICAR-Induced Glucose Transport

AMPKγ3-subunit isoform specific involvement in AICAR- and/or contraction-stimulated glucose transport was determined. Specifically, whether the R225Q mutation alters these processes in wild type, Tg-Prkag3^{wt}, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice was addressed.

Independent of nutritional status (i.e. under fasted or fed conditions) AICAR-stimulated glucose transport was significantly reduced in Tg-Prkag3^{225Q} mice, whereas this effect was totally abolished in Prkag3$^{-/-}$ mice (figure 9**)**. This establishes the AMPKγ3-subunit as a key regulator of AICAR-stimulated glucose transport and confirms that AMPKγ1- and γ2-isoforms do not compensate for the loss of γ3. The observations are consistent with the phenotype observed in AMPKα2 knock out mice (Jorgensen et al. 2004) and kinase dead form (Mu et al. 2001), since α2β2γ3 is the heterotrimer that preferentially associates in Tg-Prkag3^{225Q} mice. Reduced AICAR-stimulated glucose transport in Tg-Prkag3^{225Q} mice might directly be due to increased glycolytic muscle glycogen content (Wojtaszewski et al. 2002). In addition reduced AICAR-stimulated glucose transport in Tg-Prkag3^{225Q} mice is consistent with the loss of AMP sensitivity. AICAR-stimulated glucose transport was not fully abolished in Tg-Prkag3^{225Q} mice compared to Prkag3$^{-/-}$, thereby suggesting that the mutation might not lead to a total loss of AMP or ATP regulation. This might explain the additional increase in AICAR-induced ACC phosphorylation observed in Tg-Prkag3^{225Q} mice. In contrast to AMPKα2 knock out mice Tg-Prkag3^{225Q} mice are AICAR tolerant. However the muscle phenotype might be masked by a whole body effect of AICAR on the liver (Bergeron et al. 2001).

Contraction-stimulated glucose transport was similar between all genotypes and this response was preserved in fed and fasted mice. Contraction-stimulated glucose transport was also unaltered in AMPKα2 knock out mice (Jorgensen et al. 2004) and kinase dead form (Mu et al. 2001), respectively. Thus in our hands changes in skeletal muscle glycogen content do not seem to alter contraction-stimulated glucose transport (Derave et al. 1999, Derave et al., 2000) . Altogether these results suggest that an AMPK-independent/contraction-dependent mechanism might also support glucose transport. Intensity and nature of the contraction force generated appears to play a role (Ihlemann et al. 1999, Ihlemann et al. 2000) as well as AMPKα1-isoform specific involvement in contraction-stimulated glucose transport (Jorgensen et al. 2004).

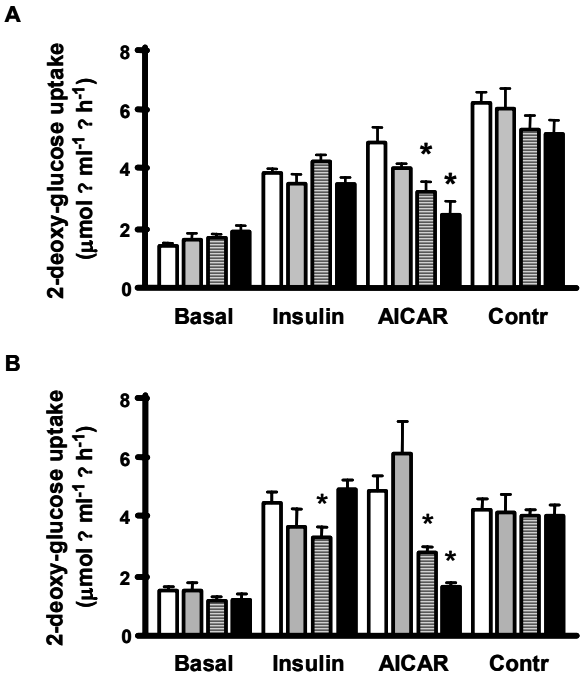

Figure 9. Nutritional status determines insulin- but not AICAR-resistance in Tg-Prkag3^{225Q} mice. Skeletal muscle glucose uptake was assessed in isolated EDL muscle under basal or after stimulation with insulin (12 nM), AICAR (2 mM), or electrically induced contraction (20 Hz for 10 min) in fed (A) or fasted (B) wild type (open bar), Tg-Prkag3^{wt} (gray bar), Tg-Prkag3^{225Q} (horizontal lines), and Prkag3$^{-/-}$ (filled bar) mice. Results are mean±SE for n=4-20 observations. *P<0.05 vs. respective basal.

The AMPKγ3-Subunit is Dispensable for AICAR-Induced Fatty Acid Oxidation

To test whether AMPKγ3-isoform is involved in regulating skeletal muscle fatty acid utilization, basal-, insulin- and AICAR-stimulated fatty acid oxidation was determined in wild-type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. Fatty acid oxidation under basal conditions was similar between all three genotypes when mice were fed a standard diet. This is consistent with γ3 playing a major role in glycolytic fibers rather than in oxidative fibers (Mahlapuu et al. 2004).

AICAR relieves insulin inhibition of fatty acid oxidation in skeletal muscle (Kaushik et al. 2001). AICAR-stimulated fatty acid oxidation was similar between genotypes, whereas insulin suppression of fatty acid oxidation was prevented in Tg-Prkag3^{225Q} mice (figure 10). This is consistent with the insulin resistance observed in Tg-Prkag3^{225Q} mice at the level of glucose transport. However results for fatty acid oxidation contrast those for ACC phosphorylation (paper IV) under basal- and AICAR-stimulated conditions, respectively in Tg-Prkag3^{225Q} mice. Recent evidence suggests that concurrent intracellular accumulation of palmitoyl CoA and phosphorylation of ACC by AMPK have stronger effects on ACC inhibition than either alone (Rubink et al. 2004). Similarly, malonyl CoA and phosphorylation of MCD are synergistically regulated (Ruderman et al. 2003). Thus the R225Q mutation might alter intracellular metabolism thereby indirectly controlling fatty acid oxidation. Moreover, dual metabolic and AMPK regulation of ACC and MCD may account for the contrasting

results observed on fatty acid oxidation and ACC phosphorylation. Furthermore there is evidence that AICAR-stimulated ACC phosphorylation and fatty acid oxidation are unrelated. Indeed AICAR-stimulated fat oxidation seems to be dependent on nutritional state, whereas AICAR-induced ACC phosphorylation is unrelated (Kaushik et al. 2001). This suggests that another kinase or phosphatase regulates ACC phosphorylation.

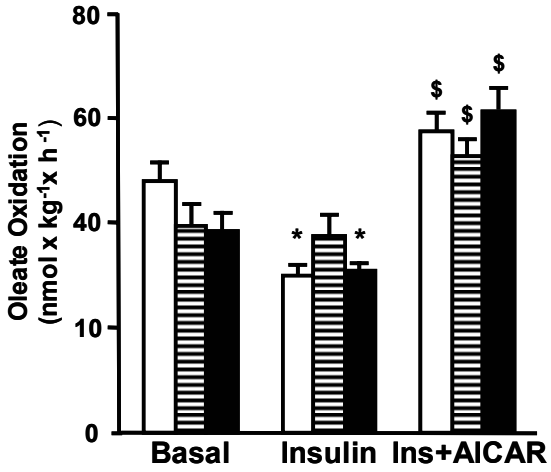

Figure 10. AMPKγ3 is dispensable for AMPK-stimulated fatty acid oxidation. Oleate oxidation was assessed in isolated EDL muscle from wild type (open bar), Tg-Prkag3^{225Q} (horizontal lines) and Prkag3$^{-/-}$ (filled bar) mice. Basal oxidation was determined in the presence of 0.3 mM oleate. Insulin (12 nM) was used to suppress oleate oxidation and observe AICAR stimulation. Results are mean±SE for n=12-18 observations. *P<0.05 vs. basal; $^{\$}$P<0.05 vs. insulin.

Fatty acid (FA) oxidation and metabolism are complex processes involving many enzymes. In skeletal muscle lipoprotein Lipase (LPL1) and CD36 allow FA release from circulating TG and FA entry into the mycocyte, respectively. Once inside the cell, FA are esterified into FA-Coenzyme A (FA CoA) and either stored back into TG or taken up by the mitochondria via Carnitine Palmitoyl Transferase (CPT1) for oxidation. Once in the mitochondria, FA-CoA can either be exported back to the cytosol via Uncoupling Protein (UCP3) or enter the tricarboxylic acid cycle following stepwise action of Hydroxy-Acetyl CoA Deshydrogenase (HAD) and citrate synthase (CS) to generate acetyl CoA and citrate, respectively. Thus multiple levels of regulation are to be considered. AMPK has been postulated to regulate gene expression and mitochondrial biogenesis (Zong et al. 2002). Thus mitochondrial function and associated lipid metabolism were determined in wild type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice by investigating relative mRNA expression of LPL1, CD36, CPT1, UCP3, HAD and CS. In Tg-Prkag3^{225Q} mice skeletal muscle CD36, UCP3 and CS were increased, whereas LPL1, CPT1 and HAD were unchanged. These observations suggest that the R225Q mutation alters lipid metabolism. Increased CS gene expression might explain the increased ACC phosphorylation noted in Tg-Prkag3^{225Q} mice (Vavvas et al. 1997). Indeed increased FA oxidation is associated with increased ACC phosphorylation and subsequent ACC inactivation. In contrast, mitochondrial gene expression in Prkag3$^{-/-}$ mice appears to be normal (figure 14). Thus the combined effect of the R225Q

mutation and subsequent altered metabolism might indirectly affect muscle mitochondrial biogenesis.

Thus the heterotrimeric complex formed by α2-, β2- and γ3-AMPK isoforms is dispensable for skeletal muscle fatty acid oxidation. However the R225Q mutation in combination with lipid oversupply appears to significantly alter mitochondrial function but this does not translate to altered fatty acid oxidation when mice are standard-fed. Challenging the mice with high fat diet might reveal such a phenotype.

The AMPKγ3-R225Q Mutation Protects Against Diet-Induced Skeletal Muscle Insulin Resistance

To test whether Tg-Prkag3^{225Q} mice are protected against diet-induced skeletal muscle insulin resistance, wild-type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice were challenged with a 55% fat-containing diet for 12 weeks and metabolic consequences were assessed (paper IV). High fat feeding does not alter the glycogen phenotype conferred by the mutation (figure 11). However TG was increased in skeletal muscle from high fat-fed wild-type and Prkag3$^{-/-}$ mice, thereby providing a mechanism for the concomitant insulin resistance developed in these models. Fatty acid oxidation is reduced in wild-type but not in Prkag3$^{-/-}$ mice, consistent with the indirect role that the γ3-subunit might play in this process.

On the other hand high fat feeding did not alter the glycogen phenotype conferred by the mutation. However, intramuscular TG content is not increased in Tg-Prkag3^{225Q} mice when fed a high fat diet. Accumulation of TG on the high fat diet is prevented by the increase in fatty acid oxidation in Tg-Prkag3^{225Q} mice, suggesting that these mice oxidize more lipids. This is consistent with the phenotype observed in mutant Hampshire pigs, whereby oxidative capacity is increased (Estrade et al. 1994, Lebret, 1999 #237, Milan, 2000 #185). Indeed, AMPK activation mediates the lipid lowering effect of Metformin in skeletal muscle and liver (Zang et al. 2004) and activation of AMPK enhances sensitivity of muscle glucose transport to insulin (Fisher et al. 2002).

High fat feeding leads to a surprising reduction in CPT-1, HAD, CS and UCP3 in Tg-Prkag3^{225Q} mice compared to standard-fed mice (figure 14). Whether this translates functionally at the level of changes in protein expression is currently unknown. Thus, under conditions where the lipid supply is increased, the R225Q mutation downregulates mitochondrial biogenesis. Nevertheless, reduced gene expression is a marker for later reduction in expression of functional proteins. Conceivably, the 12 weeks period of high fat feeding is the point at which the mutant mice are unable to compensate at the mitochondrial level. One interesting observation is that LPL1 and CD36 were also reduced, which suggests that the skeletal muscle might be in an overloaded energetic state that results in selective fatty acid directing towards the adipose tissue. However, abdominal fat accumulation as measured by fat mass in Tg-Prkag3^{225Q} mice was unchanged compared to wild-type mice. Nevertheless, the R225Q mutation might confer increased skeletal muscle metabolic flexibility that allows intracellular fuel redirection towards increased utilization. This is particularly evident when mice are fed a high fat diet and of great interest since lipid oversupply precipitates the appearance of insulin resistance and T2DM.

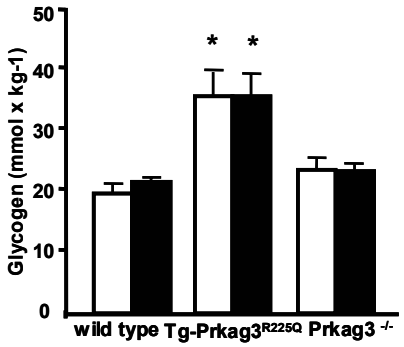

Figure 11. High fat feeding does not alter muscle glycogen content in Tg-Prkag3^{R225Q} and Prkag3$^{-/-}$ mice. Muscle glycogen content was measured in fed female mice. Gastrocnemius muscle was excised from standard (open bar) or high fat (closed bar) –fed wild type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. Results are mean±SE for 8-10 observations. P<0.01 vs. wild type in the same condition.

The AMPKγ3-R225Q Mutation Does Not Protect Against Diet-Induced Whole Body Insulin Resistance

The effect of the R225Q mutation on whole body glucose homeostasis was determined by a glucose tolerance test. Tg-Prkag3^{225Q} mice are not protected against diet induced glucose intolerance. Indeed high fat-fed Tg-Prkag3^{225Q} mice are glucose intolerant and this might be due to increased hepatic TG content (figures 12 and 13). In addition, the AMPKγ3-subunit does not appear to modulate hepatic energetic status since Prkag3$^{-/-}$ mice have normal liver TG. Thus AMPKγ3-subunit specifically regulates energy status in glycolytic skeletal muscle, whereby it prevents lipid-induced insulin resistance. AMPK has been suggested to play a central role in regulating energy homeostasis. AMPKα2$^{-/-}$ mice display whole body insulin resistance that results from increased sympathetic tone, rather than impaired skeletal muscle insulin sensitivity (Viollet et al. 2003). The current understanding is that central regulation of AMPK opposes peripheral insulin action (Andersson et al. 2004). In other words, AMPK activation in the hypothalamus results in hyperphagia and insulin resistance in rats (Namkoong et al. 2005), whereas AMPK activation prevents diet-induced muscle insulin resistance in transgenic mice. Thus the role of tissue specific isoforms becomes of paramount importance and caution must be made when modulating AMPK activity at the whole body level.

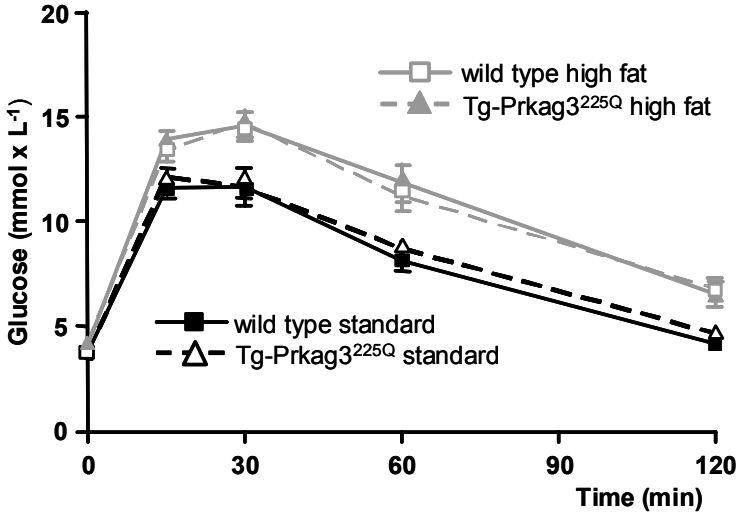

Figure 12. High fat fed Tg-Prkag3^{R225Q} mice are glucose intolerant. Glucose (2 g/kg bw) tolerance was assessed in wild type (square/solid line) and Tg-Prkag3^{225Q} (triangle/dashed line) fed a standard (black lines) or high fat (gray lines) diet for 12 weeks. Thereafter blood glucose was measured at 15, 30, 60 and 120 min. Results are mean±SE for n=15-20 mice.

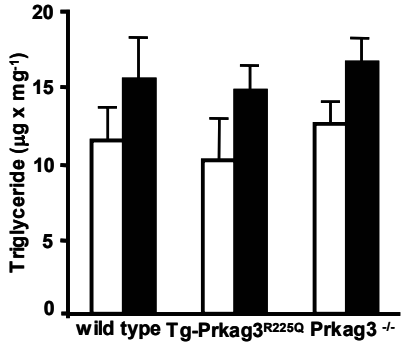

Figure 13. High fat feeding does not alter liver triglyceride content in Tg-Prkag3^{R225Q} and Prkag3$^{-/-}$ mice. Liver triglyceride content was measured in fed mice. Gastrocnemius muscle was excised from standard- (open bar) or high fat- (closed bar) fed wild type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice. Results are mean±SE for 4-6 observations.

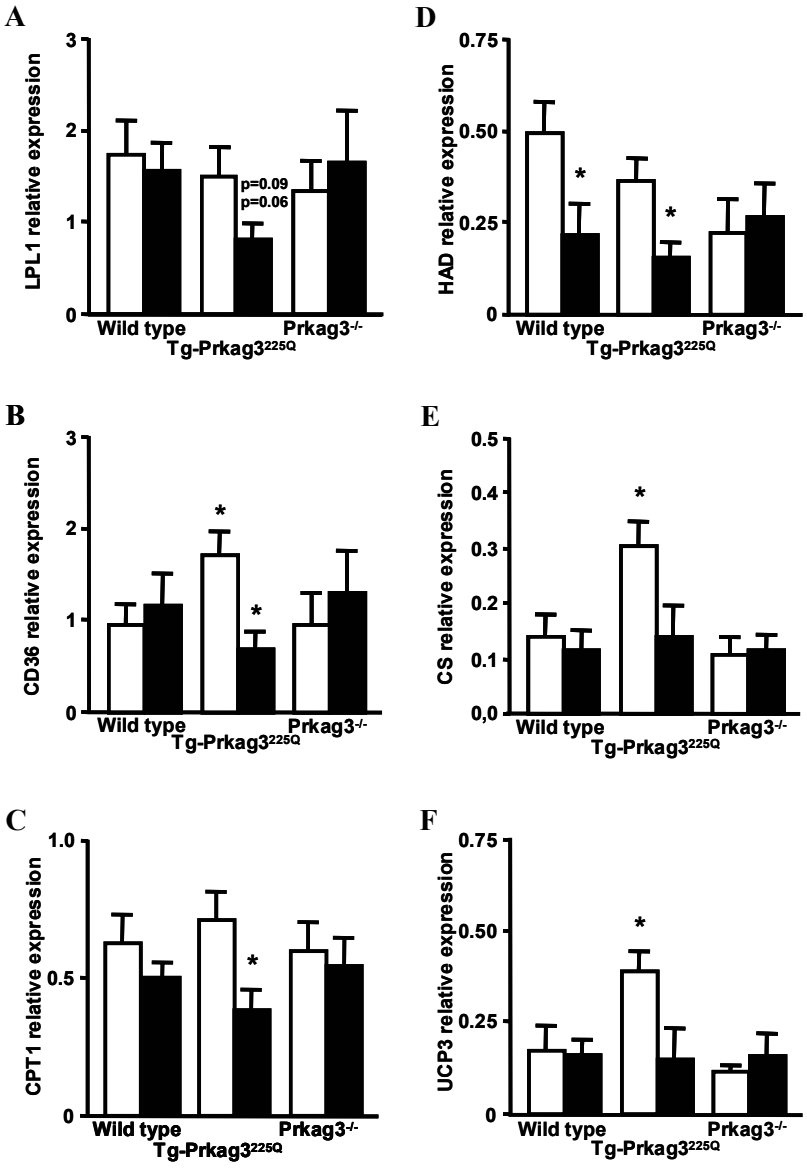

Figure 14. Lipid gene expression is upregulated in standard and downregulated in skeletal muscle from high fat fed Tg-Prkag3^{225Q} mice.
Relative mRNA expression of LPL1 (A), CD36 (B), CPT-1 (C), HAD (D), CS (E) and UCP-3 (F). Gastrocnemius muscle was excised for RNA extraction and purification. Quantification of mRNA was performed using real-time PCR with the ABI PRISM 7700 Sequence Detector System. Data is expressed relative to endogenous 36B4 gene. Results are mean±SE for n=6-10 observations. For B, C and D: P<0.05 vs. standard on same genetic background. For E and F: P<0.05 vs. wild type on same diet. For A: P=0.09 vs. wild type on the same diet and P=0.06 vs. standard on same genetic background.

Strategies to Prevent Skeletal Muscle Insulin Resistance

Skeletal muscle insulin resistance is an important pathophysiological feature of T2DM and therefore a major site of action for therapeutic strategies. Directly modulating, or bypassing defective insulin signaling may provide a mechanism to increase GLUT4 translocation to the plasma membrane and subsequent glucose transport in skeletal muscle, with the ultimate goal of restoring glucose homeostasis. Glucose transport and gene regulatory responses to GLUT4 translation are activated following insulin-dependent and -independent stimuli. In diabetic GK rat insulin-dependent PI3K signaling is defective whereas ERK1/2 response is intact. In contrast obese Zucker *fa/fa* and *ob/ob* rodents exhibit insulin resistance in PI3K and MAPK signaling, respectively. Thus dyslipidemia leads to more profound defects on insulin intracellular signaling compared to hyperglycemia. Indeed glucose may contribute to impair insulin-stimulated PI3K signaling via direct modulation of PDK-1 and PKC isoforms whereas ERK1/2 is not regulated by glucose. In addition defects in IR number and activation in morbidly obese but not hyperglycemic states, might explain defective PI3K and MAPK signaling in obese animal models. Strategies aimed to improve defective insulin signaling may prove useful for the treatment of insulin resistance in T2DM, but may be more challenging to treat insulin resistance in morbid obesity. Conversely, intact insulin-independent activation of MAPK and AMPK signaling in skeletal muscle from insulin resistant severely obese Zucker *fa/fa* rats suggests that MAPK and AMPK offer potential sites of pharmacological intervention for the regulation of glucose homeostasis. Functional analysis of AMPKγ3-specific subunit shows that AMPK activation prevents diet-induced skeletal muscle insulin resistance and provides biological validation for AMPK as a potential therapeutic target for the treatment of insulin resistance.

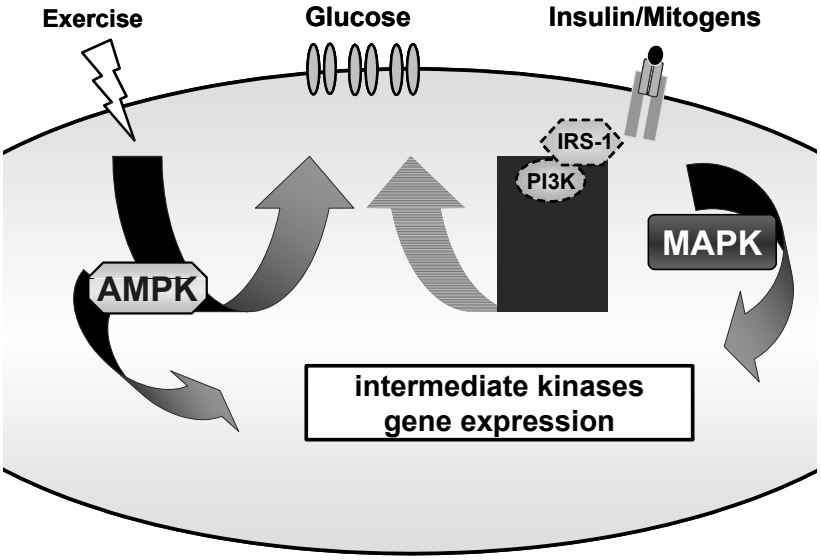

Figure 15. AMPK and MAPK: potential therapeutic targets

SUMMARY

- Insulin-stimulated defects in PI3K and PKB are not reversed by acute euglycemia and may account for skeletal muscle insulin resistance in non-obese diabetic GK rats.

- In skeletal muscle, glucose directly activates PDK-1 and downstream PKC isoforms (α/β, δ and ζ) phosphorylation through a mechanism that is independent of changes in PI3K, PKB and ERK1/2 activation.

- Insulin induces a robust increase in ERK1/2, p38-MAPK and JNK phosphorylation in a similar time- and dose-dependent manner.

- Insulin-stimulated p38-MAPK and JNK phosphorylation occur in a glycolytic skeletal muscle fiber-type-specific manner, whereas insulin-stimulated ERK1/2 phosphorylation is indistinguishable between oxidative and glycolytic fibers.

- PMA and contraction significantly increase phosphorylation of all three MAPK modules. The combined effect of insulin and contraction on p38-MAPK is reduced, whereas no additivity is observed for JNK and ERK1/2 phosphorylation.

- Insulin and PMA elicit additive effects on JNK and ERK1 phosphorylation, whereas ERK2 and p38-MAPK are phosphorylated by a kinase presumably activated by insulin and PMA signaling pathways.

- Defects in MAPK signaling account for skeletal muscle insulin resistance in *ob/ob* mice, whereas contraction- and PMA-stimulated ERK1/2 and JNK phosphorylation are unaffected by obesity driven insulin resistance.

- Reduced p38-MAPK expression may account for skeletal muscle insulin resistance in *ob/ob* mice since functional defects are observed in response to insulin-dependent and insulin-independent stimuli.

- In Zucker *fa/fa* rat hypoxia- and contraction-stimulated glucose transport and AMPKα2 activation is preserved in skeletal muscle despite severe insulin resistance.

- Contraction-stimulated AMPKα1 isoform specific defect in skeletal muscle from Zucker *fa/fa* rats is inconsequential for glucose transport activity.

- The predominant heterotrimeric AMPK complex accounting for activity contains AMPKα2β2γ3 subunits.

- The AMPKγ3 R225Q missense mutation is an activating mutation that results in increased glycolytic muscle glycogen content.

- The AMPKγ3 isoform is required for AICAR-stimulated glucose transport but is dispensable for AICAR-stimulated fatty acid oxidation.

- The AMPKγ3 R225Q mutation protects against dietary-induced skeletal muscle insulin resistance via a mechanism that prevents intramuscular triglyceride accumulation and subsequent insulin resistance, via enhanced fatty acid oxidation.

CONCLUSIONS and FUTURE PERSPECTIVES

This thesis was directed towards identifying signaling defects in PI3K and MAPK pathways that underlie skeletal muscle insulin resistance in diabetic GK rats and *ob/ob* mice. In addition insulin-independent activation of MAPK and AMPK pathways were also investigated in order to determine whether exercise-, stress- and PMA-mediated signaling cascades to glucose transport and mitogenic responses could potentially bypass defective insulin signaling in insulin resistant *ob/ob* and Zucker *fa/fa* rodents. Finally AMPK was validated as a potential target for pharmacological intervention to treat skeletal muscle insulin resistance associated with T2DM and obesity.

The mechanisms underlying glucose-induced insulin resistance is complex and appears to depend upon time and concentration factors. Mild chronic hyperglycemia, as in diabetic GK rats, impairs insulin signaling at the level of PI3K and PKB, whereas acute high glucose exposure directly modulates PDK-1 and PKC. In addition, acute euglycemia in rats chronically exposed to mild or elevated plasma glucose concentrations normalizes PKB and PKCζ, but not PI3K activation. Thus glucose effects on PKC occur within hours and are reversible, whereas PKB defects require longer exposure to mild or elevated glucose concentrations and normalization occurs within days. Finally the regulation of PI3K is independent of glycemia. To date, PKC isoforms are the only established players in hyperglycemia-induced insulin resistance. A clearer picture of isoform specific PKC dysregulation following hyperglycemia is emerging.

Glucose-induced PDK-1 activation is a key finding of this thesis. Exposure of skeletal muscle to elevated glucose concentration rapidly results in a shift in glucose metabolism towards increased storage and reduced utilization. This is very interesting in light of the glucose effects on PKB and glycogen metabolism. PDK-1 may regulate glycogen metabolism independently of PKB via GSK3 under acute hyperglycemic conditions. A better understanding of PDK-1 regulation *in vivo* is needed. To date PDK-1 defects in insulin resistant states have not been identified, however, this thesis implicated PDK-1 as a glucose-regulated target.

MAPK modules are multiproteic complex whose organization allow for transduction and/or amplification of highly specialized signals. Distinguishing the activation of distinct MAPK following extracellular stimuli is challenging. The activating mechanisms upstream of MEKK are largely undefined. Specificity in signaling might come from the distinct receptors recruited. Receptors for insulin-independent stimuli including exercise and stress are undescribed, which makes it challenging to delineate the molecular mechanisms upstream of MEKK activation. The combined effect of insulin and contraction does not elicit additive effects on JNK and ERK1/2 phosphorylation. Similarly no additivity was observed for insulin- and PMA-stimulated ERK2 and p38-MAPK phosphorylation. Thus PMA- and contraction-stimuli may share common signaling cascades with insulin to activate some but not all MAPK. Finally identification of fast twitch fiber-specific insulin-stimulated activation of p38-MAPK and JNK signaling raises the possibility of a fiber-type specific activation of MAPK on insulin sensitivity.

Contraction-stimulated ERK1/2, JNK and AMPK activation in morbidly obese insulin resistant *ob/ob* and Zucker *fa/fa* rodents is intact. Thus the signaling pathway

activated by contraction is preserved in severely insulin resistant states. Understanding the underlying mechanisms becomes of paramount importance to identify potential sites of pharmacological intervention. Differences in isoform-specific AMPK catalytic subunit activation following distinct *in vitro* contraction and *in vivo* exercise protocols may reveal selectivity in the AMPK signaling pathway. In addition understanding changes in intracellular energy status following exercise/contraction may prove informative.

Isoform-specific multimeric composition of signal transducers, as well as subsequent intracellular specialized function, is key information to specific and better drug targeting. AMPK-α2β2γ3 is the predominant heterotrimer active in glycolytic muscle *in vivo*. Functional analysis of the R225Q mutation in the AMPKγ3-isoform reveals modification of AMPK to a constitutively active kinase. The R225Q mutation in the AMPKγ3 isoform alters skeletal muscle glycogen content, thereby suggesting a key role of this subunit in glucose intracellular metabolism. Understanding the underlying mechanism for AMPK-induced glycogen accumulation is essential to understand the effect of the mutation. One hint is the possibility that AMPK directly binds to glycogen via a glycogen-binding domain on its β regulatory subunit.

Finally AMPKγ3-R225Q mutation prevents skeletal muscle, but not whole body diet-induced insulin resistance. The R225Q mutation increases lipid oxidation in the presence of lipid oversupply, which attenuates the expected accumulation of intramuscular TG content. However hepatic TG accumulation was similar between wild type, Tg-Prkag3^{225Q} and Prkag3$^{-/-}$ mice, thereby providing a mechanism for impaired glucose tolerance in Tg-Prkag3^{225Q} mice. Central and peripheral AMPK regulations oppose themselves. This, along with tissue-specific AMPK heterotrimeric composition, is key information to prevent concurrent increased peripheral tissue fuel utilization and food intake. Especially since increased food intake together with a sedentary life style contribute to the development of T2DM.

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REFERENCES

Adams, J., Z.-P. Chen, B. J. W. Van Denderen, C. J. Morton, M. W. Parker, L. A. Witters, D. Stapleton and B. E. Kemp (**2004**). "Intrasteric control of AMPK via the {gamma}1 subunit AMP allosteric regulatory site." *Protein Sci* **13**(1): 155-165.

Aguirre, V., T. Uchida, L. Yenush, R. Davis and M. F. White (**2000**). "The c-Jun NH2-terminal Kinase Promotes Insulin Resistance during Association with Insulin Receptor Substrate-1 and Phosphorylation of Ser307." *J. Biol. Chem.* **275**(12): 9047-9054.

Aguirre, V., E. D. Werner, J. Giraud, Y. H. Lee, S. E. Shoelson and M. F. White (**2002**). "Phosphorylation of Ser307 in Insulin Receptor Substrate-1 Blocks Interactions with the Insulin Receptor and Inhibits Insulin Action." *J. Biol. Chem.* **277**(2): 1531-1537.

Alessi, D. R., M. Andjelkovic, B. Caudwell, P. Cron, N. Morrice, P. Cohen and B. A. Hemmings (**1996**). "Mechanism of activation of protein kinase B by insulin and IGF-1." *Embo J* **15**(23): 6541-51.

Alessi, D. R., S. R. James, C. P. Downes, A. B. Holmes, P. R. Gaffney, C. B. Reese and P. Cohen (**1997**). "Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha." *Curr Biol* **7**(4): 261-9.

Almind, K., C. Bjorbaek, H. Vestergaard, T. Hansen, S. Echwald and O. Pedersen (**1993**). "Aminoacid polymorphisms of insulin receptor substrate-1 in non-insulin-dependent diabetes mellitus." *Lancet* **342**(8875): 828-32.

Almind, K., G. Inoue, O. Pedersen and C. R. Kahn (**1996**). "A common amino acid polymorphism in insulin receptor substrate-1 causes impaired insulin signaling. Evidence from transfection studies." *J Clin Invest* **97**(11): 2569-75.

Andersen, P. H., S. Lund, H. Vestergaard, S. Junker, B. B. Kahn and O. Pedersen (**1993**). "Expression of the major insulin regulatable glucose transporter (GLUT4) in skeletal muscle of noninsulin-dependent diabetic patients and healthy subjects before and after insulin infusion." *J Clin Endocrinol Metab* **77**(1): 27-32.

Andersson, L. (**2003**). "Identification and characterization of AMPK gamma 3 mutations in the pig." *Biochem Soc Trans* **31**(Pt 1): 232-5.

Andersson, U., K. Filipsson, C. R. Abbott, A. Woods, K. Smith, S. R. Bloom, D. Carling and C. J. Small (**2004**). "AMP-activated protein kinase plays a role in the control of food intake." *J Biol Chem* **279**(13): 12005-8.

Arad, M., D. W. Benson, A. R. Perez-Atayde, W. J. McKenna, E. A. Sparks, R. J. Kanter, K. McGarry, J. G. Seidman and C. E. Seidman (**2002**). "Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy." *J. Clin. Invest.* **109**(3): 357-362.

Araki, E., M. A. Lipes, M. E. Patti, J. C. Bruning, B. Haag, 3rd, R. S. Johnson and C. R. Kahn (**1994**). "Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene." *Nature* **372**(6502): 186-90.

Aronson, D., S. D. Dufresne and L. J. Goodyear (**1997a**). "Contractile Activity Stimulates the c-Jun NH2-terminal Kinase Pathway in Rat Skeletal Muscle." *J. Biol. Chem.* **272**(41): 25636-25640.

Aronson, D., M. A. Violan, S. D. Dufresne, D. Zangen, R. A. Fielding and L. J. Goodyear (**1997**b). "Exercise Stimulates the Mitogen-activated Protein Kinase Pathway in Human Skeletal Muscle." *J. Clin. Invest.* **99**(6): 1251-1257.

Avignon, A., K. Yamada, X. Zhou, B. Spencer, O. Cardona, S. Saba-Siddique, L. Galloway, M. L. Standaert and R. V. Farese (**1996**). "Chronic activation of protein kinase C in soleus muscles and other tissues of insulin-resistant type II diabetic Goto-Kakizaki (GK), obese/aged, and obese/Zucker rats. A mechanism for inhibiting glycogen synthesis." *Diabetes* **45**(10): 1396-404.

Azevedo, J. L., Jr., J. O. Carey, W. J. Pories, P. G. Morris and G. L. Dohm (**1995**). "Hypoxia stimulates glucose transport in insulin-resistant human skeletal muscle." *Diabetes* **44**(6): 695-8.

Bandyopadhyay, G., M. L. Standaert, L. Zhao, B. Yu, A. Avignon, L. Galloway, P. Karnam, J. Moscat and R. V. Farese (**1997**). "Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells. Transfection studies suggest a role for PKC-zeta in glucose transport." *J Biol Chem* **272**(4): 2551-8.

Bandyopadhyay, G., M. L. Standaert, M. P. Sajan, L. M. Karnitz, L. Cong, M. J. Quon and R. V. Farese (**1999**). "Dependence of Insulin-Stimulated Glucose Transporter 4 Translocation on 3-Phosphoinositide-Dependent Protein Kinase-1 and Its Target Threonine-410 in the Activation Loop of Protein Kinase C-{zeta}." *Mol Endocrinol* **13**(10): 1766-1772.

Bandyopadhyay, G., M. P. Sajan, Y. Kanoh, M. L. Standaert, M. J. Quon, B. C. Reed, I. Dikic and R. V. Farese (**2001**). "Glucose Activates Protein Kinase C-zeta /lambda through Proline-rich Tyrosine Kinase-2, Extracellular Signal-regulated Kinase, and Phospholipase D. A Novel Mechanism for Aactivating Glucose Transporter Translocation." *J. Biol. Chem.* **276**(38): 35537-35545.

Beeson, M., M. P. Sajan, M. Dizon, D. Grebenev, J. Gomez-Daspet, A. Miura, Y. Kanoh, J. Powe, G. Bandyopadhyay, M. L. Standaert and R. V. Farese (**2003**). "Activation of Protein Kinase C-{zeta} by Insulin and Phosphatidylinositol-3,4,5-(PO4)3 Is Defective in Muscle in Type 2 Diabetes and Impaired Glucose Tolerance: Amelioration by Rosiglitazone and Exercise." *Diabetes* **52**(8): 1926-1934.

Bergeron, R., R. R. Russell, III, L. H. Young, J.-M. Ren, M. Marcucci, A. Lee and G. I. Shulman (**1999**). "Effect of AMPK activation on muscle glucose metabolism in conscious rats." *Am J Physiol Endocrinol Metab* **276**(5): E938-944.

Bergeron, R., S. F. Previs, G. W. Cline, P. Perret, R. R. Russell III, L. H. Young and G. I. Shulman (**2001**). "Effect of 5-Aminoimidazole-4-Carboxamide-1-{beta}-D-Ribofuranoside Infusion on In Vivo Glucose and Lipid Metabolism in Lean and Obese Zucker Rats." *Diabetes* **50**(5): 1076-1082.

Berti, L., L. Mosthaf, G. Kroder, M. Kellerer, S. Tippmer, J. Mushack, E. Seffer, K. Seedorf and H. Haring (**1994**). "Glucose-induced translocation of protein kinase C isoforms in rat-1 fibroblasts is paralleled by inhibition of the insulin receptor tyrosine kinase." *J Biol Chem* **269**(5): 3381-6.

Bjornholm, M., Y. Kawano, M. Lehtihet and J. R. Zierath (**1997**). "Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation." *Diabetes* **46**(3): 524-7.

Boden, G., X. Chen, J. Rosner and M. Barton (**1995**). "Effects of a 48-h fat infusion on insulin secretion and glucose utilization." *Diabetes* **44**(10): 1239-42.

Bogoyevitch, M. A., I. Boehm, A. Oakley, A. J. Ketterman and R. K. Barr (**2004**). "Targeting the JNK MAPK cascade for inhibition: basic science and therapeutic potential." *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics* **1697**(1-2): 89-101.

Bollag, G. E., R. A. Roth, J. Beaudoin, D. Mochly-Rosen and D. E. Koshland, Jr. (**1986**). "Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity." *Proc Natl Acad Sci U S A* **83**(16): 5822-4.

Bouzakri, K., M. Roques, P. Gual, S. Espinosa, F. Guebre-Egziabher, J.-P. Riou, M. Laville, Y. Le Marchand-Brustel, J.-F. Tanti and H. Vidal (**2003**). "Reduced Activation of Phosphatidylinositol-3 Kinase and Increased Serine 636 Phosphorylation of Insulin Receptor Substrate-1 in Primary Culture of Skeletal Muscle Cells From Patients With Type 2 Diabetes." *Diabetes* **52**(6): 1319-1325.

Braiman, L., A. Alt, T. Kuroki, M. Ohba, A. Bak, T. Tennenbaum and S. R. Sampson (**1999**). "Protein Kinase C{delta} Mediates Insulin-Induced Glucose Transport in Primary Cultures of Rat Skeletal Muscle." *Mol Endocrinol* **13**(12): 2002-2012.

Braiman, L., A. Alt, T. Kuroki, M. Ohba, A. Bak, T. Tennenbaum and S. R. Sampson (**2001**). "Insulin Induces Specific Interaction between Insulin Receptor and Protein Kinase C{{delta}} in Primary Cultured Skeletal Muscle." *Mol Endocrinol* **15**(4): 565-574.

Brechtel, K., D. B. Dahl, J. Machann, O. P. Bachmann, I. Wenzel, T. Maier, C. D. Claussen, H. U. Haring, S. Jacob and F. Schick (**2001**). "Fast elevation of the intramyocellular lipid content in the presence of circulating free fatty acids and hyperinsulinemia: a dynamic 1H-MRS study." *Magn Reson Med* **45**(2): 179-83.

Bruss, M. D., E. B. Arias, G. E. Lienhard and G. D. Cartee (**2005**). "Increased Phosphorylation of Akt Substrate of 160 kDa (AS160) in Rat Skeletal Muscle in Response to Insulin or Contractile Activity." *Diabetes* **54**(1): 41-50.

Burgering, B. M. and P. J. Coffer (**1995**). "Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction." *Nature* **376**(6541): 599-602.

Carling, D. (**2004**). "The AMP-activated protein kinase cascade--a unifying system for energy control." *Trends Biochem Sci* **29**(1): 18-24.

Carlson, C. J., S. Koterski, R. J. Sciotti, G. B. Poccard and C. M. Rondinone (**2003**). "Enhanced Basal Activation of Mitogen-Activated Protein Kinases in Adipocytes From Type 2 Diabetes: Potential Role of p38 in the Downregulation of GLUT4 Expression " *Diabetes* **52**(3): 634-641.

Casamayor, A., N. A. Morrice and D. R. Alessi (**1999**). "Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1:

identification of five sites of phosphorylation in vivo." *Biochem J* **342 (Pt 2)**: 287-92.

Cavigelli, M., F. Dolfi, F. X. Claret and M. Karin (**1995**). "Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation." *Embo J* **14**(23): 5957-64.

Charron, M. J., F. C. Brosius, 3rd, S. L. Alper and H. F. Lodish (**1989**). "A glucose transport protein expressed predominately in insulin-responsive tissues." *Proc Natl Acad Sci U S A* **86**(8): 2535-9.

Cheatham, B., C. J. Vlahos, L. Cheatham, L. Wang, J. Blenis and C. R. Kahn (**1994**). "Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation." *Mol Cell Biol* **14**(7): 4902-11.

Chen, H., F. H. Nystrom, L. Q. Dong, Y. Li, S. Song, F. Liu and M. J. Quon (**2001**). "Insulin stimulates increased catalytic activity of phosphoinositide-dependent kinase-1 by a phosphorylation-dependent mechanism." *Biochemistry* **40**(39): 11851-9.

Chen, H. C., G. Bandyopadhyay, M. P. Sajan, Y. Kanoh, M. Standaert, R. V. Farese, Jr. and R. V. Farese (**2002**). "Activation of the ERK Pathway and Atypical Protein Kinase C Isoforms in Exercise- and Aminoimidazole-4-carboxamide- 1-beta -D-riboside (AICAR)-stimulated Glucose Transport." *J. Biol. Chem.* **277**(26): 23554-23562.

Chen, Z., J. Heierhorst, R. J. Mann, K. I. Mitchelhill, B. J. Michell, L. A. Witters, G. S. Lynch, B. E. Kemp and D. Stapleton (**1999**). "Expression of the AMP-activated protein kinase beta1 and beta2 subunits in skeletal muscle." *FEBS Lett* **460**(2): 343-8.

Chen, Z., T. B. Gibson, F. Robinson, L. Silvestro, G. Pearson, B. Xu, A. Wright, C. Vanderbilt and M. H. Cobb (**2001**). "MAP kinases." *Chem Rev* **101**(8): 2449-76.

Chibalin, A. V., M. Yu, J. W. Ryder, X. M. Song, D. Galuska, A. Krook, H. Wallberg-Henriksson and J. R. Zierath (**2000**). "Exercise-induced changes in expression and activity of proteins involved in insulin signal transduction in skeletal muscle: Differential effects on insulin-receptor substrates 1 and 2." *PNAS* **97**(1): 38-43.

Chin, J. E., M. Dickens, J. M. Tavare and R. A. Roth (**1993**). "Overexpression of protein kinase C isoenzymes alpha, beta I, gamma, and epsilon in cells overexpressing the insulin receptor. Effects on receptor phosphorylation and signaling." *J Biol Chem* **268**(9): 6338-47.

Chin, E. R., E. N. Olson, J. A. Richardson, Q. Yang, C. Humphries, J. M. Shelton, H. Wu, W. Zhu, R. Bassel-Duby and R. S. Williams (**1998**). "A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type." *Genes Dev.* **12**(16): 2499-2509.

Cho, H., J. Mu, J. K. Kim, J. L. Thorvaldsen, Q. Chu, E. B. Crenshaw, III, K. H. Kaestner, M. S. Bartolomei, G. I. Shulman and M. J. Birnbaum (**2001**). "Insulin Resistance and a Diabetes Mellitus-Like Syndrome in Mice Lacking the Protein Kinase Akt2 (PKBbeta)." *Science* **292**(5522): 1728-1731.

Chow, C.-W., C. Dong, R. A. Flavell and R. J. Davis (**2000**). "c-Jun NH2-Terminal Kinase Inhibits Targeting of the Protein Phosphatase Calcineurin to NFATc1." *Mol. Cell. Biol.* **20**(14): 5227-5234.

Chua, S. C., Jr., W. K. Chung, X. S. Wu-Peng, Y. Zhang, S. M. Liu, L. Tartaglia and R. L. Leibel (**1996**). "Phenotypes of mouse diabetes and rat fatty due to mutations in the OB (leptin) receptor." *Science* **271**(5251): 994-6.

Coleman, D. L. (**1978**). "Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice." *Diabetologia* **14**(3): 141-8.

Crouse, J. A., G. E. Elliott, T. L. Burgess, L. Chiu, L. Bennett, J. Moore, M. Nicolson and R. E. Pacifici (**1998**). "Altered Cell Surface Expression and Signaling of Leptin Receptors Containing the fatty Mutation." *J. Biol. Chem.* **273**(29): 18365-18373.

Cuenda, A., J. Rouse, Y. N. Doza, R. Meier, P. Cohen, T. F. Gallagher, P. R. Young and J. C. Lee (**1995**). "SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1." *FEBS Lett* **364**(2): 229-33.

da Silva, B. A., C. Bjorbak, S. Uotani and J. S. Flier (**1998**). "Functional Properties of Leptin Receptor Isoforms Containing the Gln->Pro Extracellular Domain Mutation of the Fatty Rat." *Endocrinology* **139**(9): 3681-3690.

Deak, M., A. D. Clifton, L. M. Lucocq and D. R. Alessi (**1998**). "Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB." *Embo J* **17**(15): 4426-41.

DeFronzo, R. A., R. C. Bonadonna and E. Ferrannini (**1992**). "Pathogenesis of NIDDM. A balanced overview." *Diabetes Care* **15**(3): 318-68.

Dekker, L. V. and P. J. Parker (**1994**). "Protein kinase C--a question of specificity." *Trends Biochem Sci* **19**(2): 73-7.

Derave, W., S. Lund, G. D. Holman, J. Wojtaszewski, O. Pedersen and E. A. Richter (**1999**). "Contraction-stimulated muscle glucose transport and GLUT-4 surface content are dependent on glycogen content." *Am J Physiol Endocrinol Metab* 277(6): E1103-1110.

Derave, W., H. Ai, J. Ihlemann, L. Witters, S. Kristiansen, E. Richter and T. Ploug (**2000**). "Dissociation of AMP-activated protein kinase activation and glucose transport in contracting slow-twitch muscle." *Diabetes* **49**(8): 1281-1287.

Dimitrakoudis, D., T. Ramlal, S. Rastogi, M. Vranic and A. Klip (**1992**). "Glycaemia regulates the glucose transporter number in the plasma membrane of rat skeletal muscle." *Biochem J* **284 (Pt 2)**: 341-8.

Downward, J. (**1995**). "Signal transduction. A target for PI(3) kinase." *Nature* **376**(6541): 553-4.

Downward, J. (**1996**). "Control of ras activation." *Cancer Surv* **27**: 87-100.

Eckel, J., A. Wirdeier, L. Herberg and H. Reinauer (**1985**). "Insulin resistance in the heart: studies on isolated cardiocytes of genetically obese Zucker rats." *Endocrinology* **116**(4): 1529-34.

Egawa, K., H. Maegawa, K. Shi, T. Nakamura, T. Obata, T. Yoshizaki, K. Morino, S. Shimizu, Y. Nishio, E. Suzuki and A. Kashiwagi (**2002**). "Membrane Localization of 3-Phosphoinositide-dependent Protein Kinase-1 Stimulates Activities of Akt and Atypical Protein Kinase C but Does Not Stimulate Glucose Transport and Glycogen Synthesis in 3T3-L1 Adipocytes." *J. Biol. Chem.* **277**(41): 38863-38869.

Eriksson, B. O., P. D. Gollnick and B. Saltin (**1973**). "Muscle metabolism and enzyme activities after training in boys 11-13 years old." *Acta Physiol Scand* **87**(4): 485-97.

Estrade, M., X. Vignon, E. Rock and G. Monin (**1993**). "Glycogen hyperaccumulation in white muscle fibres of RN- carrier pigs. A biochemical and ultrastructural study." *Comp Biochem Physiol B* **104**(2): 321-6.

Estrade, M., S. Ayoub, A. Talmant and G. Monin (**1994**). "Enzyme activities of glycogen metabolism and mitochondrial characteristics in muscles of RN- carrier pigs (Sus scrofa domesticus)." *Comp Biochem Physiol Biochem Mol Biol* **108**(3): 295-301.

Filippis, A., S. Clark and J. Proietto (**1997**). "Increased flux through the hexosamine biosynthesis pathway inhibits glucose transport acutely by activation of protein kinase C." *Biochem J* **324 (Pt 3)**: 981-5.

Fisher, J. S., J. Gao, D.-H. Han, J. O. Holloszy and L. A. Nolte (**2002**). "Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin." *Am J Physiol Endocrinol Metab* **282**(1): E18-23.

Folli, F., M. J. Saad, J. M. Backer and C. R. Kahn (**1993**). "Regulation of phosphatidylinositol 3-kinase activity in liver and muscle of animal models of insulin-resistant and insulin-deficient diabetes mellitus." *J Clin Invest* **92**(4): 1787-94.

Formisano, P., F. Oriente, F. Fiory, M. Caruso, C. Miele, M. A. Maitan, F. Andreozzi, G. Vigliotta, G. Condorelli and F. Beguinot (**2000**). "Insulin-Activated Protein Kinase Cbeta Bypasses Ras and Stimulates Mitogen-Activated Protein Kinase Activity and Cell Proliferation in Muscle Cells." *Mol. Cell. Biol.* **20**(17): 6323-6333.

Franke, T. F., S. I. Yang, T. O. Chan, K. Datta, A. Kazlauskas, D. K. Morrison, D. R. Kaplan and P. N. Tsichlis (**1995**). "The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase." *Cell* **81**(5): 727-36.

Franke, T. F., D. R. Kaplan, L. C. Cantley and A. Toker (**1997**). "Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate." *Science* **275**(5300): 665-8.

Fryer, L. G. D., A. Parbu-Patel and D. Carling (**2002**). "The Anti-diabetic Drugs Rosiglitazone and Metformin Stimulate AMP-activated Protein Kinase through Distinct Signaling Pathways." *J. Biol. Chem.* **277**(28): 25226-25232.

Fujii, N., T. Hayashi, M. F. Hirshman, J. T. Smith, S. A. Habinowski, L. Kaijser, J. Mu, O. Ljungqvist, M. J. Birnbaum, L. A. Witters, A. Thorell and L. J. Goodyear (**2000**). "Exercise induces isoform-specific increase in 5'AMP-activated protein kinase activity in human skeletal muscle." *Biochem Biophys Res Commun* **273**(3): 1150-5.

Gao, Z., X. Zhang, A. Zuberi, D. Hwang, M. J. Quon, M. Lefevre and J. Ye (**2004**). "Inhibition of Insulin Sensitivity by Free Fatty Acids Requires Activation of Multiple Serine Kinases in 3T3-L1 Adipocytes." *Mol Endocrinol* **18**(8): 2024-2034.

Garvey, W. T., L. Maianu, J. A. Hancock, A. M. Golichowski and A. Baron (**1992**). "Gene expression of GLUT4 in skeletal muscle from insulin-resistant patients with obesity, IGT, GDM, and NIDDM." *Diabetes* **41**(4): 465-75.

Garvey, W. T., L. Maianu, J. H. Zhu, G. Brechtel-Hook, P. Wallace and A. D. Baron (**1998**). "Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance." *J Clin Invest* **101**(11): 2377-86.

Gollob, M. H., M. S. Green, A. S.-L. Tang, T. Gollob, A. Karibe, A.-S. Hassan, F. Ahmad, R. Lozado, G. Shah, L. Fananapazir, L. L. Bachinski, T. Tapscott, O. Gonzales, D. Begley, S. Mohiddin and R. Roberts (**2001**). "Identification of a Gene Responsible for Familial Wolff-Parkinson-White Syndrome." *N Engl J Med* **344**(24): 1823-1831.

Goodyear, L. J., F. Giorgino, T. W. Balon, G. Condorelli and R. J. Smith (**1995**). "Effects of contractile activity on tyrosine phosphoproteins and PI 3-kinase activity in rat skeletal muscle." *Am J Physiol Endocrinol Metab* **268**(5): E987-995.

Goodyear, L. J., F. Giorgino, L. A. Sherman, J. Carey, R. J. Smith and G. L. Dohm (**1995**). "Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects." *J Clin Invest* **95**(5): 2195-204.

Goodyear, L. J., P. Y. Chang, D. J. Sherwood, S. D. Dufresne and D. E. Moller (**1996**). "Effects of exercise and insulin on mitogen-activated protein kinase signaling pathways in rat skeletal muscle." *Am J Physiol Endocrinol Metab* **271**(2): E403-408.

Goto, Y., M. Kakizaki and N. Masaki (**1976**). "Production of spontaneous diabetic rats by repetition of selective breeding." *Tohoku J Exp Med* **119**(1): 85-90.

Goto, Y., K. Suzuki, T. Ono, M. Sasaki and T. Toyota (**1988**). "Development of diabetes in the non-obese NIDDM rat (GK rat)." *Adv Exp Med Biol* **246**: 29-31.

Gray, S., I. Idris, K. R. Davis and R. Donnelly (**2003**). "Increased skeletal muscle expression of PKCtheta but not PKCalpha mRNA in type 2 diabetes: inverse relationship with insulin sensitivity." *Eur J Clin Invest* **33**(11): 983-987.

Greiwe, J. S., R. C. Hickner, P. A. Hansen, S. B. Racette, M. M. Chen and J. O. Holloszy (**1999**). "Effects of endurance exercise training on muscle glycogen accumulation in humans." *J Appl Physiol* **87**(1): 222-226.

Griffin, M. E., M. J. Marcucci, G. W. Cline, K. Bell, N. Barucci, D. Lee, L. J. Goodyear, E. W. Kraegen, M. F. White and G. I. Shulman (**1999**). "Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade." *Diabetes* **48**(6): 1270-4.

Grillo, S., T. Grémeaux, Y. Le Marchand-Brustel and J.-F. Tanti (**1999**). "Potential role of 3-phosphoinositide-dependent protein kinase 1 (PDK1) in insulin-stimulated glucose transporter 4 translocation in adipocytes." *FEBS Letters* **461**(3): 277-279.

Grundleger, M. L. and S. W. Thenen (**1982**). "Decreased insulin binding, glucose transport, and glucose metabolism in soleus muscle of rats fed a high fat diet." *Diabetes* **31**(3): 232-7.

Guma, A., J. R. Zierath, H. Wallberg-Henriksson and A. Klip (**1995**). "Insulin induces translocation of GLUT-4 glucose transporters in human skeletal muscle." *Am J Physiol* **268**(4 Pt 1): E613-22.

Hager, S. R., A. L. Jochen and R. K. Kalkhoff (**1991**). "Insulin resistance in normal rats infused with glucose for 72 h." *Am J Physiol* **260**(3 Pt 1): E353-62.

Halaas, J. L., K. S. Gajiwala, M. Maffei, S. L. Cohen, B. T. Chait, D. Rabinowitz, R. L. Lallone, S. K. Burley and J. M. Friedman (**1995**). "Weight-reducing effects of the plasma protein encoded by the obese gene." *Science* **269**(5223): 543-6.

Hamilton, S. R., D. Stapleton, J. B. O'Donnell, Jr., J. T. Kung, S. R. Dalal, B. E. Kemp and L. A. Witters (**2001**). "An activating mutation in the gamma1 subunit of the AMP-activated protein kinase." *FEBS Lett* **500**(3): 163-8.

Hanada, M., J. Feng and B. A. Hemmings (**2004**). "Structure, regulation and function of PKB/AKT--a major therapeutic target." *Biochim Biophys Acta* **1697**(1-2): 3-16.

Hansen, P., E. Gulve, J. Gao, J. Schluter, M. Mueckler and J. Holloszy (**1995**). "Kinetics of 2-deoxyglucose transport in skeletal muscle: effects of insulin and contractions." *Am J Physiol* **268**(1 Pt 1): C30-5.

Hansen, P. A., J. A. Corbett and J. O. Holloszy (**1997**). "Phorbol esters stimulate muscle glucose transport by a mechanism distinct from the insulin and hypoxia pathways." *Am J Physiol* **273**(1 Pt 1): E28-36.

Hardie, D. G. and D. Carling (**1997**). "The AMP-activated protein kinase--fuel gauge of the mammalian cell?" *Eur J Biochem* **246**(2): 259-73.

Hardie, D. G. (**2003**). "Minireview: the AMP-activated protein kinase cascade: the key sensor of cellular energy status." *Endocrinology* **144**(12): 5179-83.

Hayashi, T., M. Hirshman, E. Kurth, W. Winder and L. Goodyear (**1998**). "Evidence for 5' AMP-activated protein kinase mediation of the effect of muscle contraction on glucose transport." *Diabetes* **47**(8): 1369-1373.

Hayashi, T., M. Hirshman, N. Fujii, S. Habinowski, L. Witters and L. Goodyear (**2000**). "Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism." *Diabetes* **49**(4): 527-531.

Herbst, J. J., G. C. Andrews, L. G. Contillo, D. H. Singleton, P. E. Genereux, E. M. Gibbs and G. E. Lienhard (**1995**). "Effect of the activation of phosphatidylinositol 3-kinase by a thiophosphotyrosine peptide on glucose transport in 3T3-L1 adipocytes." *J Biol Chem* **270**(43): 26000-5.

Hickey, M. S., J. O. Carey, J. L. Azevedo, J. A. Houmard, W. J. Pories, R. G. Israel and G. L. Dohm (**1995**). "Skeletal muscle fiber composition is related to adiposity and in vitro glucose transport rate in humans." *Am J Physiol Endocrinol Metab* **268**(3): E453-457.

Hirosumi, J., G. Tuncman, L. Chang, C. Z. Gorgun, K. T. Uysal, K. Maeda, M. Karin and G. S. Hotamisligil (**2002**). "A central role for JNK in obesity and insulin resistance." *Nature* **420**(6913): 333-336.

Holmes, B. F., E. J. Kurth-Kraczek and W. W. Winder (**1999**). "Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle." *J Appl Physiol* **87**(5): 1990-1995.

Houmard, J. A., M. H. Shinebarger, P. L. Dolan, N. Leggett-Frazier, R. K. Bruner, M. R. McCammon, R. G. Israel and G. L. Dohm (**1993**). "Exercise training

increases GLUT-4 protein concentration in previously sedentary middle-aged men." *Am J Physiol* **264**(6 Pt 1): E896-901.

Hug, H. and T. F. Sarre (**1993**). "Protein kinase C isoenzymes: divergence in signal transduction?" *Biochem J* **291 (Pt 2)**: 329-43.

Hughes, V. A., M. A. Fiatarone, R. A. Fielding, B. B. Kahn, C. M. Ferrara, P. Shepherd, E. C. Fisher, R. R. Wolfe, D. Elahi and W. J. Evans (**1993**). "Exercise increases muscle GLUT-4 levels and insulin action in subjects with impaired glucose tolerance." *Am J Physiol* **264**(6 Pt 1): E855-62.

I**glesias, M. A.**, J.-M. Ye, G. Frangioudakis, A. K. Saha, E. Tomas, N. B. Ruderman, G. J. Cooney and E. W. Kraegen (**2002**). "AICAR Administration Causes an Apparent Enhancement of Muscle and Liver Insulin Action in Insulin-Resistant High-Fat-Fed Rats." *Diabetes* **51**(10): 2886-2894.

Ihlemann, J., T. Ploug, Y. Hellsten and H. Galbo (**1999**). "Effect of tension on contraction-induced glucose transport in rat skeletal muscle." *Am J Physiol Endocrinol Metab* **277**(2): E208-214.

Ihlemann, J., T. Ploug, Y. Hellsten and H. Galbo (**2000**). "Effect of stimulation frequency on contraction-induced glucose transport in rat skeletal muscle." *Am J Physiol Endocrinol Metab* **279**(4): E862-867.

Inoue, H., W. Ogawa, M. Ozaki, S. Haga, M. Matsumoto, K. Furukawa, N. Hashimoto, Y. Kido, T. Mori, H. Sakaue, K. Teshigawara, S. Jin, H. Iguchi, R. Hiramatsu, D. LeRoith, K. Takeda, S. Akira and M. Kasuga (**2004**). "Role of STAT-3 in regulation of hepatic gluconeogenic genes and carbohydrate metabolism in vivo." **10**(2): 168-174.

Ishizuka, T., A. Miura, K. Kajita, M. Ishizawa, M. Kimura, Y. Huang, Y. Kawai, H. Morita, Y. Uno and K. Yasuda (**2001**). "Differential effect of PKC isoform on insulin- and phorbol ester-stimulated glucose uptake mechanism in rat adipocytes." *IUBMB Life* **51**(5): 299-304.

Itani, S., Q. Zhou, W. Pories, K. MacDonald and G. Dohm (**2000**). "Involvement of protein kinase C in human skeletal muscle insulin resistance and obesity." *Diabetes* **49**(8): 1353-1358.

Itani, S., W. Pories, K. MacDonald and G. Dohm (**2001**). "Increased protein kinase C theta in skeletal muscle of diabetic patients." *Metabolism - Clinical and Experimental* **50**(5): 553-557.

Jakobsen, S. N., D. G. Hardie, N. Morrice and H. E. Tornqvist (**2001**). "5'-AMP-activated Protein Kinase Phosphorylates IRS-1 on Ser-789 in Mouse C2C12 Myotubes in Response to 5-Aminoimidazole-4-carboxamide Riboside." *J. Biol. Chem.* **276**(50): 46912-46916.

Janknecht, R., W. H. Ernst and A. Nordheim (**1995**). "SAP1a is a nuclear target of signaling cascades involving ERKs." *Oncogene* **10**(6): 1209-16.

Jiang, Z. Y., Q. L. Zhou, K. A. Coleman, M. Chouinard, Q. Boese and M. P. Czech (**2003**). "Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing." *PNAS* **100**(13): 7569-7574.

Jorgensen, S. B., B. Viollet, F. Andreelli, C. Frosig, J. B. Birk, P. Schjerling, S. Vaulont, E. A. Richter and J. F. Wojtaszewski (**2004**). "Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-

aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle." *J Biol Chem* **279**(2): 1070-9.

Kahn, B. and O. Pedersen (**1993**). "Suppression of GLUT4 expression in skeletal muscle of rats that are obese from high fat feeding but not from high carbohydrate feeding or genetic obesity." *Endocrinology* **132**(1): 13-22.

Kahn, C. R., D. M. Neville, Jr. and J. Roth (**1973**). "Insulin-Receptor Interaction in the Obese-Hyperglycemic Mouse. A Model of Insulin Resistanec." *J. Biol. Chem.* **248**(1): 244-250.

Kane, S., H. Sano, S. C. H. Liu, J. M. Asara, W. S. Lane, C. C. Garner and G. E. Lienhard (**2002**). "A Method to Identify Serine Kinase Substrates. Akt Phosphorylates a Novel Adipocytes Protein with A Rab GTPase-Activating Protein (GAP) Domain." *J. Biol. Chem.* **277**(25): 22115-22118.

Kanety, H., R. Feinstein, M. Z. Papa, R. Hemi and A. Karasik (**1995**). "Tumor Necrosis Factor alpha-induced Phosphorylation of Insulin Receptor Substrate-1 (IRS-1)." *J. Biol. Chem.* **270**(40): 23780-23784.

Kanoh, Y., G. Bandyopadhyay, M. P. Sajan, M. L. Standaert and R. V. Farese (**2000**). "Thiazolidinedione treatment enhances insulin effects on protein kinase C-zeta /lambda activation and glucose transport in adipocytes of nondiabetic and Goto-Kakizaki type II diabetic rats." *J Biol Chem* **275**(22): 16690-6.

Kanoh, Y., G. Bandyopadhyay, M. P. Sajan, M. L. Standaert and R. V. Farese (**2001**). "Rosiglitazone, Insulin Treatment, and Fasting Correct Defective Activation of Protein Kinase C-{{zeta}}/{{lambda}} by Insulin in Vastus Lateralis Muscles and Adipocytes of Diabetic Rats." *Endocrinology* **142**(4): 1595-1605.

Kasuga, M., Y. Fujita-Yamaguchi, D. L. Blithe and C. R. Kahn (**1983**). "Tyrosine-specific protein kinase activity is associated with the purified insulin receptor." *Proc Natl Acad Sci U S A* **80**(8): 2137-41.

Kaushik, V. K., M. E. Young, D. J. Dean, T. G. Kurowski, A. K. Saha and N. B. Ruderman (**2001**). "Regulation of fatty acid oxidation and glucose metabolism in rat soleus muscle: effects of AICAR." *Am J Physiol Endocrinol Metab* **281**(2): E335-40.

Kawano, Y., J. Rincon, A. Soler, J. W. Ryder, L. A. Nolte, J. R. Zierath and H. Wallberg-Henriksson (**1999**). "Changes in glucose transport and protein kinase Cbeta(2) in rat skeletal muscle induced by hyperglycaemia." *Diabetologia* **42**(9): 1071-9.

Kelley, D. E. and J. A. Simoneau (**1994**). "Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus." *J Clin Invest* **94**(6): 2349-56.

Kemp, B. E., D. Stapleton, D. J. Campbell, Z. P. Chen, S. Murthy, M. Walter, A. Gupta, J. J. Adams, F. Katsis, B. van Denderen, I. G. Jennings, T. Iseli, B. J. Michell and L. A. Witters (**2003**). "AMP-activated protein kinase, super metabolic regulator." *Biochem Soc Trans* **31**(Pt 1): 162-8.

Kikkawa, R., M. Haneda, T. Uzu, D. Koya, T. Sugimoto and Y. Shigeta (**1994**). "Translocation of protein kinase C alpha and zeta in rat glomerular mesangial cells cultured under high glucose conditions." *Diabetologia* **37**(8): 838-41.

Kim, J. K., J. J. Fillmore, M. J. Sunshine, B. Albrecht, T. Higashimori, D.-W. Kim, Z.-X. Liu, T. J. Soos, G. W. Cline, W. R. O'Brien, D. R. Littman and G. I.

Shulman (**2004**). "PKC-{theta} knockout mice are protected from fat-induced insulin resistance." *J. Clin. Invest.* **114**(6): 823-827.

Kim, B., P. S. Leventhal, A. R. Saltiel and E. L. Feldman (**1997**). "Insulin-like growth factor-I-mediated neurite outgrowth in vitro requires mitogen-activated protein kinase activation." *J Biol Chem* **272**(34): 21268-73.

Kim, Y. B., S. E. Nikoulina, T. P. Ciaraldi, R. R. Henry and B. B. Kahn (**1999**). "Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes." *J Clin Invest* **104**(6): 733-41.

Kim, Y. B., G. I. Shulman and B. B. Kahn (**2002**). "Fatty acid infusion selectively impairs insulin action on Akt1 and protein kinase C lambda /zeta but not on glycogen synthase kinase-3." *J Biol Chem* **277**(36): 32915-22.

Kim, Y. B., K. Kotani, T. P. Ciaraldi, R. R. Henry and B. B. Kahn (**2003**). "Insulin-Stimulated Protein Kinase C {lambda}/{zeta} Activity Is Reduced in Skeletal Muscle of Humans With Obesity and Type 2 Diabetes: Reversal With Weight Reduction." *Diabetes* **52**(8): 1935-1942.

Kohn, A. D., S. A. Summers, M. J. Birnbaum and R. A. Roth (**1996**). "Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation." *J Biol Chem* **271**(49): 31372-8.

Koistinen, H. A., A. V. Chibalin and J. R. Zierath (**2003**). "Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from Type 2 diabetic patients." *Diabetologia* **46**(10): 1324-8.

Koistinen, H. A., D. Galuska, A. V. Chibalin, J. Yang, J. R. Zierath, G. D. Holman and H. Wallberg-Henriksson (**2003**). "5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes." *Diabetes* **52**(5): 1066-72.

Kraegen, E. W., D. E. James, L. H. Storlien, K. M. Burleigh and D. J. Chisholm (**1986**). "In vivo insulin resistance in individual peripheral tissues of the high fat fed rat: assessment by euglycaemic clamp plus deoxyglucose administration." *Diabetologia* **29**(3): 192-8.

Kroder, G., B. Bossenmaier, M. Kellerer, E. Capp, B. Stoyanov, A. Muhlhofer, L. Berti, H. Horikoshi, A. Ullrich and H. Haring (**1996**). "Tumor Necrosis Factor-alpha - and Hyperglycemia-induced Insulin Resistance . Evidence for Different Mechanisms and Different Effects on Insulin Signaling." *J. Clin. Invest.* **97**(6): 1471-1477.

Krook, A., Y. Kawano, X. M. Song, S. Efendic, R. A. Roth, H. Wallberg-Henriksson and J. R. Zierath (**1997**). "Improved glucose tolerance restores insulin-stimulated Akt kinase activity and glucose transport in skeletal muscle from diabetic Goto-Kakizaki rats." *Diabetes* **46**(12): 2110-4.

Krook, A., R. A. Roth, X. J. Jiang, J. R. Zierath and H. Wallberg-Henriksson (**1998**). "Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects." *Diabetes* **47**(8): 1281-6.

Krook, A., M. Björnholm, D. Galuska, X. Jiang, R. Fahlman, M. Myers, H. Wallberg-Henriksson and J. Zierath (**2000**). "Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients." *Diabetes* **49**(2): 284-292.

Kurowski, T. G., Y. Lin, Z. Luo, P. N. Tsichlis, M. G. Buse, S. J. Heydrick and N. B. Ruderman (**1999**). "Hyperglycemia inhibits insulin activation of Akt/protein kinase B but not phosphatidylinositol 3-kinase in rat skeletal muscle." *Diabetes* **48**(3): 658-63.

Laakso, M., M. Malkki, P. Kekalainen, J. Kuusisto and S. S. Deeb (**1994**). "Insulin receptor substrate-1 variants in non-insulin-dependent diabetes." *J Clin Invest* 94(3): 1141-6.

Laemmli, U. K. (**1970**). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." *Nature* **227**(5259): 680-5.

Lee, Y. H., J. Giraud, R. J. Davis and M. F. White (**2003**). "c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade." *J Biol Chem* **278**(5): 2896-902.

Leevers, S. J., H. F. Paterson and C. J. Marshall (**1994**). "Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane." *Nature* **369**(6479): 411-4.

Le Marchand-Brustel, Y., P. Gual, T. Gremeaux, T. Gonzalez, R. Barres and J. F. Tanti (**2003**). "Fatty acid-induced insulin resistance: role of insulin receptor substrate 1 serine phosphorylation in the retroregulation of insulin signalling." *Biochem Soc Trans* **31**(Pt 6): 1152-6.

Letiges, M., M. Plomann, M. L. Standaert, G. Bandyopadhyay, M. P. Sajan, Y. Kanoh and R. V. Farese (**2002**). "Knockout of PKC{alpha} Enhances Insulin Signaling Through PI3K." *Mol Endocrinol* **16**(4): 847-858.

Leturque, A., C. Postic, P. Ferre and J. Girard (**1991**). "Nutritional regulation of glucose transporter in muscle and adipose tissue of weaned rats." *Am J Physiol Endocrinol Metab* **260**(4): E588-593.

Li, Y., T. J. Soos, X. Li, J. Wu, M. Degennaro, X. Sun, D. R. Littman, M. J. Birnbaum and R. D. Polakiewicz (**2004**). "Protein kinase C Theta inhibits insulin signaling by phosphorylating IRS1 at Ser(1101)." *J Biol Chem* **279**(44): 45304-7.

Lim, C. P. and X. Cao (**1999**). "Serine Phosphorylation and Negative Regulation of Stat3 by JNK." *J. Biol. Chem.* **274**(43): 31055-31061.

Mahlapuu, M., C. Johansson, K. Lindgren, G. Hjalm, B. R. Barnes, A. Krook, J. R. Zierath, L. Andersson and S. Marklund (**2004**). "Expression profiling of the {gamma}-subunit isoforms of AMP-activated protein kinase suggests a major role for {gamma}3 in white skeletal muscle." *Am J Physiol Endocrinol Metab* **286**(2): E194-200.

Marais, R., Y. Light, H. F. Paterson and C. J. Marshall (**1995**). "Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation." *Embo J* **14**(13): 3136-45.

Markuns, J. F., J. F. Wojtaszewski and L. J. Goodyear (**1999**). "Insulin and exercise decrease glycogen synthase kinase-3 activity by different mechanisms in rat skeletal muscle." *J Biol Chem* **274**(35): 24896-900.

Marques, F., M. E. Crespo, Z. I. Silva and M. Bicho (**2000**). "Insulin and high glucose modulation of phosphatase and reductase enzymes in the human erythrocytes: a comparative analysis in normal and diabetic states." *Diabetes Res Clin Pract* **47**(3): 191-8.

Marshall, B. A. and M. M. Mueckler (**1994**). "Differential effects of GLUT-1 or GLUT-4 overexpression on insulin responsiveness in transgenic mice." *Am J Physiol Endocrinol Metab* **267**(5): E738-744.

Milan, D., J.-T. Jeon, C. Looft, V. Amarger, A. Robic, M. Thelander, C. Rogel-Gaillard, S. Paul, N. Iannuccelli, L. Rask, H. Ronne, K. Lundström, N. Reinsch, J. Gellin, E. Kalm, P. L. Roy, P. Chardon and L. Andersson (**2000**). "A Mutation in PRKAG3 Associated with Excess Glycogen Content in Pig Skeletal Muscle." *Science* **288**(5469): 1248-1251.

Mora, A., D. Komander, D. M. van Aalten and D. R. Alessi (**2004**). "PDK1, the master regulator of AGC kinase signal transduction." *Semin Cell Dev Biol* **15**(2): 161-70.

Mordes, J. P. and A. A. Rossini (**1981**). "Animal models of diabetes." *Am J Med* **70**(2): 353-60.

Moxham, C. M., A. Tabrizchi, R. J. Davis and C. C. Malbon (**1996**). "Jun N-terminal kinase mediates activation of skeletal muscle glycogen synthase by insulin in vivo." *J Biol Chem* **271**(48): 30765-73.

Mu, J., J. Brozinick, Joseph T., O. Valladares, M. Bucan and M. J. Birnbaum (**2001**). "A Role for AMP-Activated Protein Kinase in Contraction- and Hypoxia-Regulated Glucose Transport in Skeletal Muscle." *Molecular Cell* **7**(5): 1085-1094.

Mu, J., E. R. Barton and M. J. Birnbaum (**2003**). "Selective suppression of AMP-activated protein kinase in skeletal muscle: update on 'lazy mice'." *Biochem Soc Trans* **31**(Pt 1): 236-41.

Musi, N., N. Fujii, M. F. Hirshman, I. Ekberg, S. Froberg, O. Ljungqvist, A. Thorell and L. J. Goodyear (**2001**). "AMP-Activated Protein Kinase (AMPK) Is Activated in Muscle of Subjects With Type 2 Diabetes During Exercise." *Diabetes* **50**(5): 921-927.

Musi, N., T. Hayashi, N. Fujii, M. F. Hirshman, L. A. Witters and L. J. Goodyear (**2001**). "AMP-activated protein kinase activity and glucose uptake in rat skeletal muscle." *Am J Physiol Endocrinol Metab* **280**(5): E677-684.

Musi, N., M. F. Hirshman, J. Nygren, M. Svanfeldt, P. Bavenholm, O. Rooyackers, G. Zhou, J. M. Williamson, O. Ljunqvist, S. Efendic, D. E. Moller, A. Thorell and L. J. Goodyear (**2002**). "Metformin Increases AMP-Activated Protein Kinase Activity in Skeletal Muscle of Subjects With Type 2 Diabetes " *Diabetes* **51**(7): 2074-2081.

Musi, N. and L. J. Goodyear (**2003**). "AMP-activated protein kinase and muscle glucose uptake." *Acta Physiol Scand* **178**(4): 337-345.

Musi, N., H. Yu and L. J. Goodyear (**2003**). "AMP-activated protein kinase regulation and action in skeletal muscle during exercise." *Biochem Soc Trans* **31**(Pt 1): 191-5.

Nakamura, K., C. J. Zhou, J. Parente and C. S. Chew (**1996**). "Parietal cell MAP kinases: multiple activation pathways." *Am J Physiol* **271**(4 Pt 1): G640-9.

Namkoong, C., M. S. Kim, P. G. Jang, S. M. Han, H. S. Park, E. H. Koh, W. J. Lee, J. Y. Kim, I. S. Park, J. Y. Park and K. U. Lee (**2005**). "Enhanced Hypothalamic AMP-Activated Protein Kinase Activity Contributes to Hyperphagia in Diabetic Rats." *Diabetes* **54**(1): 63-68.

Newton, A. C. (**1995**). "Protein kinase C: structure, function, and regulation." *J Biol Chem* **270**(48): 28495-8.

Nielsen, J. N., J. Vissing, J. F. P. Wojtaszewski, R. G. Haller, N. Begum and E. A. Richter (**2002**). "Decreased insulin action in skeletal muscle from patients with McArdle's disease." *Am J Physiol Endocrinol Metab* **282**(6): E1267-1275.

Nishizuka, Y. (**1995**). "Protein kinase C and lipid signaling for sustained cellular responses." *Faseb J* **9**(7): 484-96.

Oku, A., M. Nawano, K. Ueta, T. Fujita, I. Umebayashi, K. Arakawa, T. Kano-Ishihara, A. Saito, M. Anai, M. Funaki, M. Kikuchi, Y. Oka and T. Asano (**2001**). "Inhibitory effect of hyperglycemia on insulin-induced Akt/protein kinase B activation in skeletal muscle." *Am J Physiol Endocrinol Metab* **280**(5): E816-824.

Olsen, G. S. and B. F. Hansen (**2002**). "AMP kinase activation ameliorates insulin resistance induced by free fatty acids in rat skeletal muscle." *Am J Physiol Endocrinol Metab* **283**(5): E965-970.

Osman, A. A., J. Hancock, D. G. Hunt, J. L. Ivy and L. J. Mandarino (**2001**). "Exercise training increases ERK2 activity in skeletal muscle of obese Zucker rats." *J Appl Physiol* **90**(2): 454-460.

Park, J., M. M. Hill, D. Hess, D. P. Brazil, J. Hofsteenge and B. A. Hemmings (**2001**). "Identification of Tyrosine Phosphorylation Sites on 3-Phosphoinositide-dependent Protein Kinase-1 and Their Role in Regulating Kinase Activity." *J. Biol. Chem.* **276**(40): 37459-37471.

Parker, P. J. (**1999**). "Inhibition of protein kinase C--do we, can we, and should we?" *Pharmacol Ther* **82**(2-3): 263-7.

Pearson, G., F. Robinson, T. Beers Gibson, B.-e. Xu, M. Karandikar, K. Berman and M. H. Cobb (**2001**). "Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions." *Endocr Rev* **22**(2): 153-183.

Pelleymounter, M. A., M. J. Cullen, M. B. Baker, R. Hecht, D. Winters, T. Boone and F. Collins (**1995**). "Effects of the obese gene product on body weight regulation in ob/ob mice." *Science* **269**(5223): 540-3.

Perrini, S., J. Henriksson, J. R. Zierath and U. Widegren (**2004**). "Exercise-Induced Protein Kinase C Isoform-Specific Activation in Human Skeletal Muscle." *Diabetes* **53**(1): 21-24.

Picarel-Blanchot, F., C. Berthelier, D. Bailbe and B. Portha (**1996**). "Impaired insulin secretion and excessive hepatic glucose production are both early events in the diabetic GK rat." *Am J Physiol Endocrinol Metab* **271**(4): E755-762.

Puceat, M. and G. Vassort (**1996**). "Signalling by protein kinase C isoforms in the heart." *Mol Cell Biochem* **157**(1-2): 65-72.

Ren, J. M., B. A. Marshall, M. M. Mueckler, M. McCaleb, J. M. Amatruda and G. I. Shulman (**1995**). "Overexpression of Glut4 protein in muscle increases basal and insulin-stimulated whole body glucose disposal in conscious mice." *J Clin Invest* **95**(1): 429-32.

Roden, M., T. B. Price, G. Perseghin, K. F. Petersen, D. L. Rothman, G. W. Cline and G. I. Shulman (**1996**). "Mechanism of free fatty acid-induced insulin resistance in humans." *J Clin Invest* **97**(12): 2859-65.

Rossetti, L., G. I. Shulman, W. Zawalich and R. A. DeFronzo (**1987**). "Effect of chronic hyperglycemia on in vivo insulin secretion in partially pancreatectomized rats." *J Clin Invest* **80**(4): 1037-44.

Rossetti, L., D. Smith, G. I. Shulman, D. Papachristou and R. A. DeFronzo (**1987**). "Correction of hyperglycemia with phlorizin normalizes tissue sensitivity to insulin in diabetic rats." *J Clin Invest* **79**(5): 1510-5.

Rothman, D. L., I. Magnusson, G. Cline, D. Gerard, C. R. Kahn, R. G. Shulman and G. I. Shulman (**1995**). "Decreased muscle glucose transport/phosphorylation is an early defect in the pathogenesis of non-insulin-dependent diabetes mellitus." *Proc Natl Acad Sci U S A* **92**(4): 983-7.

Rubink, D. S. and W. W. Winder (**2004**). "Effect of Phosphorylation by AMP-activated Protein Kinase on Palmitoyl-CoA Inhibition of Skeletal Muscle Acetyl-CoA Carboxylase." *J Appl Physiol:* 00621.2004.

Ruderman, N. B., H. Park, V. K. Kaushik, D. Dean, S. Constant, M. Prentki and A. K. Saha (**2003**). "AMPK as a metabolic switch in rat muscle, liver and adipose tissue after exercise." *Acta Physiol Scand* **178**(4): 435-442.

Ryder, J. W., R. Bassel-Duby, E. N. Olson and J. R. Zierath (**2003**). "Skeletal Muscle Reprogramming by Activation of Calcineurin Improves Insulin Action on Metabolic Pathways." *J. Biol. Chem.* **278**(45): 44298-44304.

Saad, M. J., F. Folli, J. A. Kahn and C. R. Kahn (**1993**). "Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats." *J Clin Invest* **92**(4): 2065-72.

Salt, I., J. W. Celler, S. A. Hawley, A. Prescott, A. Woods, D. Carling and D. G. Hardie (**1998**). "AMP-activated protein kinase: greater AMP dependence, and preferential nuclear localization, of complexes containing the alpha2 isoform." *Biochem J* **334 (Pt 1)**: 177-87.

Sano, H., S. Kane, E. Sano, C. P. Miinea, J. M. Asara, W. S. Lane, C. W. Garner and G. E. Lienhard (**2003**). "Insulin-stimulated Phosphorylation of a Rab GTPase-activating Protein Regulates GLUT4 Translocation." *J. Biol. Chem.* **278**(17): 14599-14602.

Schmitz-Peiffer, C., C. L. Browne, N. D. Oakes, A. Watkinson, D. J. Chisholm, E. W. Kraegen and T. J. Biden (**1997**). "Alterations in the expression and cellular localization of protein kinase C isozymes epsilon and theta are associated with insulin resistance in skeletal muscle of the high-fat-fed rat." *Diabetes* **46**(2): 169-78.

Schmitz-Peiffer, C., D. L. Craig and T. J. Biden (**1999**). "Ceramide Generation Is Sufficient to Account for the Inhibition of the Insulin-stimulated PKB Pathway in C2C12 Skeletal Muscle Cells Pretreated with Palmitate." *J. Biol. Chem.* **274**(34): 24202-24210.

Schmitz-Peiffer, C. (**2000**). "Signalling aspects of insulin resistance in skeletal muscle: mechanisms induced by lipid oversupply." *Cellular Signalling* **12**(9-10): 583-594.

Skolnik, E. Y., A. Batzer, N. Li, C. H. Lee, E. Lowenstein, M. Mohammadi, B. Margolis and J. Schlessinger (**1993**). "The function of GRB2 in linking the insulin receptor to Ras signaling pathways." *Science* **260**(5116): 1953-5.

Soll, A. H., C. R. Kahn and D. M. Neville, Jr (1975). "Insulin binding to liver plasm membranes in the obese hyperglycemic (ob/ob) mouse. Demonstration of a decreased number of functionally normal receptors." *J. Biol. Chem.* **250**(12): 4702-4707.

Somwar, R., M. Perreault, S. Kapur, C. Taha, G. Sweeney, T. Ramlal, D. Y. Kim, J. Keen, C. H. Cote, A. Klip and A. Marette (**2000**). "Activation of p38 mitogen-activated protein kinase alpha and beta by insulin and contraction in rat skeletal muscle: potential role in the stimulation of glucose transport." *Diabetes* **49**(11): 1794-800.

Somwar, R., D. Y. Kim, G. Sweeney, C. Huang, W. Niu, C. Lador, T. Ramlal and A. Klip (**2001**). "GLUT4 translocation precedes the stimulation of glucose uptake by insulin in muscle cells: potential activation of GLUT4 via p38 mitogen-activated protein kinase." *Biochem J* **359**(Pt 3): 639-49.

Song, X. M., Y. Kawano, A. Krook, J. W. Ryder, S. Efendic, R. A. Roth, H. Wallberg-Henriksson and J. R. Zierath (**1999**). "Muscle fiber type-specific defects in insulin signal transduction to glucose transport in diabetic GK rats." *Diabetes* **48**(3): 664-70.

Song, X. M., M. Fiedler, D. Galuska, J. W. Ryder, M. Fernstrom, A. V. Chibalin, H. Wallberg-Henriksson and J. R. Zierath (**2002**). "5-Aminoimidazole-4-carboxamide ribonucleoside treatment improves glucose homeostasis in insulin-resistant diabetic (ob/ob) mice." *Diabetologia* **45**(1): 56-65.

Standaert, M. L., A. Avignon, K. Yamada, G. Bandyopadhyay and R. V. Farese (**1996**). "The phosphatidylinositol 3-kinase inhibitor, wortmannin, inhibits insulin-induced activation of phosphatidylcholine hydrolysis and associated protein kinase C translocation in rat adipocytes." *Biochem J* 313 (Pt 3): 1039-46.

Standaert, M. L., G. Bandyopadhyay, L. Galloway, J. Soto, Y. Ono, U. Kikkawa, R. V. Farese and M. Leitges (**1999**). "Effects of Knockout of the Protein Kinase C {beta} Gene on Glucose Transport and Glucose Homeostasis." *Endocrinology* **140**(10): 4470-4477.

Stapleton, D., K. I. Mitchelhill, G. Gao, J. Widmer, B. J. Michell, T. Teh, C. M. House, C. S. Fernandez, T. Cox, L. A. Witters and B. E. Kemp (**1996**). "Mammalian AMP-activated Protein Kinase Subfamily." *J. Biol. Chem.* **271**(2): 611-614.

Steinberg, G. R., J. W. E. Rush and D. J. Dyck (**2003**). "AMPK expression and phosphorylation are increased in rodent muscle after chronic leptin treatment." *Am J Physiol Endocrinol Metab* **284**(3): E648-654.

Storlien, L. H., D. A. Pan, A. D. Kriketos and L. A. Baur (**1993**). "High fat diet-induced insulin resistance. Lessons and implications from animal studies." *Ann N Y Acad Sci* **683**: 82-90.

Storlien, L. H., L. A. Baur, A. D. Kriketos, D. A. Pan, G. J. Cooney, A. B. Jenkins, G. D. Calvert and L. V. Campbell (**1996**). "Dietary fats and insulin action." *Diabetologia* **39**(6): 621-31.

Tanti, J., T. Gremeaux, E. van Obberghen and Y. Le Marchand-Brustel (**1994**). "Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling." *J. Biol. Chem.* **269**(8): 6051-6057.

Tomas, E., Y.-S. Lin, Z. Dagher, A. Saha, Z. Luo, Y. Ido and N. B. Ruderman (**2002**). "Hyperglycemia and Insulin Resistance: Possible Mechanisms." *Ann NY Acad Sci* **967**(1): 43-51.

Tomas, E., T.-S. Tsao, A. K. Saha, H. E. Murrey, C. c. Zhang, S. I. Itani, H. F. Lodish and N. B. Ruderman (**2002**). "Enhanced muscle fat oxidation and glucose transport by ACRP30 globular domain: Acetyl-CoA carboxylase inhibition and AMP-activated protein kinase activation." *PNAS* **99**(25): 16309-16313.

Tremblay, F., C. Lavigne, H. Jacques and A. Marette (**2001**). "Defective Insulin-Induced GLUT4 Translocation in Skeletal Muscle of High Fat-Fed Rats Is Associated With Alterations in Both Akt/Protein Kinase B and Atypical Protein Kinase C ({zeta}/{lambda}) Activities." *Diabetes* **50**(8): 1901-1910.

Tsakiridis, T., E. Tsiani, P. Lekas, A. Bergman, V. Cherepanov, C. Whiteside and G. P. Downey (**2001**). "Insulin, insulin-like growth factor-I, and platelet-derived growth factor activate extracellular signal-regulated kinase by distinct pathways in muscle cells." *Biochem Biophys Res Commun* **288**(1): 205-11.

Vaag, A., J. E. Henriksen and H. Beck-Nielsen (**1992**). "Decreased insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non-insulin-dependent diabetes mellitus." *J Clin Invest* **89**(3): 782-8.

Vavvas, D., A. Apazidis, A. K. Saha, J. Gamble, A. Patel, B. E. Kemp, L. A. Witters and N. B. Ruderman (**1997**). "Contraction-induced Changes in Acetyl-CoA Carboxylase and 5'-AMP-activated Kinase in Skeletal Muscle." *J. Biol. Chem.* 272(20): 13255-13261.

Villena, J. A., B. Viollet, F. Andreelli, A. Kahn, S. Vaulont and H. S. Sul (**2004**). "Induced adiposity and adipocyte hypertrophy in mice lacking the AMP-activated protein kinase-alpha2 subunit." *Diabetes* **53**(9): 2242-9.

Viollet, B., F. Andreelli, S. B. Jorgensen, C. Perrin, D. Flamez, J. Mu, J. F. Wojtaszewski, F. C. Schuit, M. Birnbaum, E. Richter, R. Burcelin and S. Vaulont (**2003**). "Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models." *Biochem Soc Trans* **31**(Pt 1): 216-9.

Viollet, B., F. Andreelli, S. B. Jorgensen, C. Perrin, A. Geloen, D. Flamez, J. Mu, C. Lenzner, O. Baud, M. Bennoun, E. Gomas, G. Nicolas, J. F. Wojtaszewski, A. Kahn, D. Carling, F. C. Schuit, M. J. Birnbaum, E. A. Richter, R. Burcelin and S. Vaulont (**2003**). "The AMP-activated protein kinase alpha2 catalytic subunit controls whole-body insulin sensitivity." *J Clin Invest* **111**(1): 91-8.

Vogt, B., C. Muhlbacher, J. Carrascosa, B. Obermaier-Kusser, E. Seffer, J. Mushack, D. Pongratz and H. U. Haring (**1992**). "Subcellular distribution of GLUT 4 in the skeletal muscle of lean type 2 (non-insulin-dependent) diabetic patients in the basal state." *Diabetologia* **35**(5): 456-63.

Wallberg-Henriksson, H., N. Zetan and J. Henriksson (**1987**). "Reversibility of decreased insulin-stimulated glucose transport capacity in diabetic muscle with in vitro incubation. Insulin is not required." *J Biol Chem* **262**(16): 7665-71.

Way, K. J., N. Katai and G. L. King (**2001**). "Protein kinase C and the development of diabetic vascular complications." *Diabet Med* **18**(12): 945-959.

White, M. F. (**1997**). "The insulin signalling system and the IRS proteins." *Diabetologia* **40 Suppl 2**: S2-17.

Whitmarsh, A. J., S. H. Yang, M. S. Su, A. D. Sharrocks and R. J. Davis (**1997**). "Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors." *Mol Cell Biol* **17**(5): 2360-71.

Wijkander, J., T. R. Landstrom, V. Manganiello, P. Belfrage and E. Degerman (**1998**). "Insulin-induced phosphorylation and activation of phosphodiesterase 3B in rat adipocytes: possible role for protein kinase B but not mitogen-activated protein kinase or p70 S6 kinase." *Endocrinology* **139**(1): 219-27.

Winder, W. W. and B. F. Holmes (**2000**). "Insulin stimulation of glucose uptake fails to decrease palmitate oxidation in muscle if AMPK is activated." *J Appl Physiol* **89**(6): 2430-2437.

Winder, W. W. (**2001**). "Energy-sensing and signaling by AMP-activated protein kinase in skeletal muscle." *J Appl Physiol* **91**(3): 1017-1028.

Wojtaszewski, J. F. P., J. Lynge, A. B. Jakobsen, L. J. Goodyear and E. A. Richter (**1999**). "Differential regulation of MAP kinase by contraction and insulin in skeletal muscle: metabolic implications." *Am J Physiol Endocrinol Metab* **277**(4): E724-732.

Wojtaszewski, J. F. P., P. Nielsen, B. F. Hansen, E. A. Richter and B. Kiens (**2000**). "Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle." *J Physiol (Lond)* **528**(1): 221-226.

Wojtaszewski, J. F., M. Mourtzakis, T. Hillig, B. Saltin and H. Pilegaard (**2002**). "Dissociation of AMPK activity and ACCbeta phosphorylation in human muscle during prolonged exercise." *Biochem Biophys Res Commun* **298**(3): 309-16.

Wojtaszewski, J. F. P., S. B. Jorgensen, Y. Hellsten, D. G. Hardie and E. A. Richter (**2002**). "Glycogen-Dependent Effects of 5-Aminoimidazole-4-Carboxamide (AICA)-Riboside on AMP-Activated Protein Kinase and Glycogen Synthase Activities in RatSkeletal Muscle " *Diabetes* **51**(2): 284-292.

Wojtaszewski, J. F., J. N. Nielsen, S. B. Jorgensen, C. Frosig, J. B. Birk and E. A. Richter (**2003**). "Transgenic models--a scientific tool to understand exercise-induced metabolism: the regulatory role of AMPK (5'-AMP-activated protein kinase) in glucose transport and glycogen synthase activity in skeletal muscle." *Biochem Soc Trans* **31**(Pt 6): 1290-4.

Woods, A., I. Salt, J. Scott, D. G. Hardie and D. Carling (**1996**). "The alpha1 and alpha2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro." *FEBS Lett* **397**(2-3): 347-51.

Woods, A., S. R. Johnstone, K. Dickerson, F. C. Leiper, L. G. Fryer, D. Neumann, U. Schlattner, T. Wallimann, M. Carlson and D. Carling (**2003**). "LKB1 is the upstream kinase in the AMP-activated protein kinase cascade." *Curr Biol* **13**(22): 2004-8.

Wretman, C., U. Widegren, A. Lionikas, H. Westerblad and J. Henriksson (**2000**). "Differential activation of mitogen-activated protein kinase signalling pathways

by isometric contractions in isolated slow- and fast-twitch rat skeletal muscle." *Acta Physiol Scand* **170**(1): 45-9.

Xing, J., D. D. Ginty and M. E. Greenberg (**1996**). "Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase." *Science* **273**(5277): 959-63.

Yamashita, T., T. Murakami, M. Iida, M. Kuwajima and K. Shima (**1997**). "Leptin receptor of Zucker fatty rat performs reduced signal transduction." *Diabetes* **46**(6): 1077-80.

Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, K. Eto, Y. Akanuma, P. Froguel, F. Foufelle, P. Ferre, D. Carling, S. Kimura, R. Nagai, B. B. Kahn and T. Kadowaki (**2002**). "Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase." *Nat Med* **8**(11): 1288-95.

Yki-Jarvinen, H., E. Helve and V. A. Koivisto (**1987**). "Hyperglycemia decreases glucose uptake in type I diabetes." *Diabetes* **36**(8): 892-6.

Yki-Jarvinen, H., K. Sahlin, J. M. Ren and V. A. Koivisto (**1990**). "Localization of rate-limiting defect for glucose disposal in skeletal muscle of insulin-resistant type I diabetic patients." *Diabetes* **39**(2): 157-67.

Zang, M., A. Zuccollo, X. Hou, D. Nagata, K. Walsh, H. Herscovitz, P. Brecher, N. B. Ruderman and R. A. Cohen (**2004**). "AMP-activated Protein Kinase Is Required for the Lipid-lowering Effect of Metformin in Insulin-resistant Human HepG2 Cells." *J. Biol. Chem.* **279**(46): 47898-47905.

Zeigerer, A., M. K. McBrayer and T. E. McGraw (**2004**). "Insulin Stimulation of GLUT4 Exocytosis, but Not Its Inhibition of Endocytosis, Is Dependent on RabGAP AS160." *Mol. Biol. Cell* **15**(10): 4406-4415.

Zhao, M., L. New, V. V. Kravchenko, Y. Kato, H. Gram, F. di Padova, E. N. Olson, R. J. Ulevitch and J. Han (**1999**). "Regulation of the MEF2 family of transcription factors by p38." *Mol Cell Biol* **19**(1): 21-30.

Zierath, J. R., D. Galuska, L. A. Nolte, A. Thorne, J. S. Kristensen and H. Wallberg-Henriksson (**1994**). "Effects of glycaemia on glucose transport in isolated skeletal muscle from patients with NIDDM: in vitro reversal of muscular insulin resistance." *Diabetologia* **37**(3): 270-7.

Zierath, J. R., L. He, A. Guma, E. Odegoard Wahlstrom, A. Klip and H. Wallberg-Henriksson (**1996**). "Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM." *Diabetologia* **39**(10): 1180-9.

Zierath, J. R., K. L. Houseknecht, L. Gnudi and B. B. Kahn (**1997**). "High-fat feeding impairs insulin-stimulated GLUT4 recruitment via an early insulin-signaling defect." *Diabetes* **46**(2): 215-23.

Zong, H., J. M. Ren, L. H. Young, M. Pypaert, J. Mu, M. J. Birnbaum and G. I. Shulman (**2002**). "AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation." *PNAS* **99**(25): 15983-15987.